# AN INTRODUCTION TO THE CHEMISTRY OF PLANT PRODUCTS

P. HAAS
AND
T. G. HILL

V.12.
Extraction of oils from seeds
&c seeds last section

# AN INTRODUCTION TO THE CHEMISTRY OF PLANT PRODUCTS

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TO THE

# CHEMISTRY OF PLANT PRODUCTS

## VOL. I. ON THE NATURE AND SIGNIFICANCE OF THE COMMONER ORGANIC COMPOUNDS OF PLANTS

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*WITH DIAGRAMS*

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## PREFACE TO THE THIRD EDITION.

The necessity for a third edition has afforded an opportunity for making certain changes in the arrangement of the subject matter. In order to give the more purely physiological aspect of the subject fuller treatment, without at the same time unduly increasing the size of the volume, the work now appears in two parts. Volume I. is essentially the same in scope as the earlier editions and deals primarily with the more chemical side of the subject: a sufficiency of plant physiology has, however, been retained to make the account reasonably complete and to preserve the character of the work. Volume II., which is in preparation, will be devoted to more purely physiological problems, and will contain some of the matter previously found in the original volume.

The present volume has been brought up to date as far as is possible; some portions have been rewritten, Section VIII. for example, and in other sections a certain amount of rearrangement has been deemed advisable.

P. H.
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*October*, 1920.

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## PREFACE TO THE SECOND EDITION.

The great advances made in the chemistry of Plant Pigments since the issue of the first edition have necessitated the re-writing of the section dealing with this subject. For the rest, we have confined ourselves to making a few minor additions and corrections and adding a number of further references to the literature.

P. H.
T. G. H.

*July*, 1916.

## PREFACE TO THE FIRST EDITION.

The importance to the botanist of a working knowledge of chemistry can hardly be overestimated, since vegetable physiology is replete with problems awaiting solution by the combined application of botanical and chemical methods.

Teachers of vegetable physiology, however, not infrequently find that their students' knowledge is deficient in just those branches of chemistry which are of particular importance to the botanist, which is, no doubt, largely due to the fact that those compounds which are of interest to the botanist do not necessarily fit into the scheme of instruction of the chemist.

The present work is an attempt to provide such students, who are assumed to have some acquaintance with chemistry, with an introductory account of the chemistry and biological significance of some of the more important substances occurring in plants.

In compiling this book various sources of information have been laid under contribution, and although the point of view is, in the main, purely chemical and botanical, the economic aspect has not been lost sight of, and, where possible, mention has been made of the uses of plant products and of the manufacturing processes employed in their preparation.

P. H.
T. G. H.

*December*, 1912.

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# SECTION I.

## FATS, OILS, AND WAXES.

In ordinary parlance, no clear distinction is made in the use of the terms fat and wax, which are applied more or less indiscriminately to any solid substances which have a greasy feeling to the touch and do not mix with water. Chemically, however, there is a marked difference between the two classes; the fats are compounds of the trihydric alcohol glycerol, whereas the waxes are compounds of the higher monohydric alcohols, such as cetyl alcohol $C_{16}H_{33}OH$, myristic alcohol $C_{30}H_{61}OH$, and cholesterol $C_{27}H_{45}OH$.

The tendency to rely on physical properties only, and to regard waxes as having generally a harder consistency than fats has given rise to several cases of incorrect nomenclature. For example, wool fat and spermaceti being compounds of cholesterol and cetyl alcohol are in reality waxes, though they are usually regarded as fats, whereas the substance ordinarily known as Japan wax is actually a fat, since it is a compound of glycerol.

The term oil, as used in the ordinary sense to imply a liquid which is immiscible with water, must not be taken to have any chemical significance, since substances having this physical property are found in almost every class of chemical compound. Used in connexion with fats, the term oil simply implies a fat that is liquid at ordinary temperatures; any solid fat on melting becomes an oil, and, on the other hand, any fatty oil on solidifying becomes a fat.

### OCCURRENCE.

Fats are very widely distributed in the vegetable kingdom, the most common being the glycerides of oleic, palmitic, and stearic acids; especially are they found in reproductive bodies

such as spores and seeds. Not only are the spores, both sexual and asexual of very many Algæ and the majority of the Fungi characterized by the presence of oil, but also the thallus: the filaments of *Vaucheria*, for instance, contain much oil; and of the Fungi, the sclerotia of *Claviceps purpurea* (ergot) may contain as much as 60 per cent, whilst the mycelium of *Lactarius deliciosus* contains about 6 per cent.

The fats of the Fungi are rich in fatty acids associated with lecithins and ergosterins.

In Angiosperms they are widely distributed, especially in seeds where they may replace the carbohydrates as a reserve food-material and are not uncommonly associated with protein reserves; to mention a few examples, colza oil is obtained from the seeds of *Brassica Napus*, palm oil from the fruits of *Elaeis guineensis*, cotton-seed oil from *Gossypium herbaceum*, linseed oil from *Linum usitatissimum*, olive oil from the sarcocarp of *Olea europæa*, castor oil from the seeds of *Ricinus*, and cacao butter from the fruits of *Theobroma*.

Oils of lesser economic importance occur in the fruits or seeds of the sunflower, almond, hemp, willow and many other plants.

The amount of oil present in such structures may be quite considerable, thus in the kernel of the Brazil nut nearly 70 per cent may obtain, and in the almond about 54 per cent.

Oils also occur in the vegetative organs to a greater or lesser extent; substances of an oily nature are found in association with the chloroplasts and, in some cases, to a relatively large extent, e.g. in *Strelitzia*; sometimes it is present as a definite reserve food-material as in the tubers of *Cyperus esculentus*, where it is associated with starch, and in the roots of some orchids.

This particular form of food reserve is doubly of value since its presence may lessen the danger arising from drought, and also more energy can be stored up in the form of oil than in an equal bulk of carbohydrate; in this connexion may be mentioned the fact that in some cases the appearance of oil may be transient, thus in some trees the starch stored up in the parenchyma of the stem may be converted into fat during the winter's cold; the starch, however, reappears on a rise in temperature. Also fat or fat-like substances may appear in

the leaves of evergreen plants during the winter months. The fat-like substances, according to Meyer,* who studied *Vinca*, *Taxus* and *Ilex*, do not show a seasonal variation in amount, but continually increase with the age of the leaf. These phenomena are similar to those which will be mentioned in connexion with the conversion of starch into sugar under the influence of low temperature (p. 132).

The majority of vegetable fats are fluid at ordinary temperatures; a few, however, are solid, for instance, cacao butter and the fat in the seeds of *Myristica*.

### INDUSTRIAL USES OF VEGETABLE FATS AND OILS.

Economically, fats are of considerable value, being used for food, illumination, lubrication, soap manufacture, and for a variety of other purposes.

The following is a brief consideration of some of the more important industrial uses of the commoner fats and oils of vegetable origin.

OLIVE OIL is extracted from the fleshy pericarp of the fruit of the olive, *Olea europaea*, by pressure. The best quality oil, which is expressed without the application of heat, is used for food; lower grade oils, obtained by extracting the residues from the presses with fat solvents, such as carbon disulphide or light petroleum, are used in the manufacture of soap (see p. 5).

COTTON-SEED OIL is extracted from the seeds of *Gossypium herbaceum* by pressing them at a temperature of about 90°; the crude brown oil is purified by treatment with caustic soda which removes the free fatty acids, colouring matter and other impurities. After purification the oil is light yellow in colour. It is used for the manufacture of soap and rubber substitutes.

COCO-NUT OIL is obtained from the ripe seeds of *Cocos nucifera* and *Cocos butyracea* by pressure; the dried endosperms, known as Copra, are imported into Europe and the oil extracted from them is commonly known as Copra oil. Soaps made from coco-nut oil have the property of absorbing large

* Meyer: "Ber. deut. bot. Gesells.," 1918, 36, 5; see also Tuttle: "Ann. Bot.," 1919, 33, 201.

quantities of salt solutions and can, therefore, be used for washing with sea-water.

PALM OIL which occurs in the fruit of *Elaeis guineensis* is, when pure, a colourless substance of the consistency of lard; on exposure to air it readily turns yellow, but the colour can be removed by oxidation by means of a current of air. It has a faint odour resembling that of violets. Both coco-nut oil and palm oil in the crude state contain free fatty acids which can, however, be removed by treatment with alcohol. When so purified they are employed as substitutes for butter under the name of vegetable butter or palmine, etc.

RAPE OIL or COLZA OIL is a thick, yellowish oil obtained from the seeds of *Brassica Rapa* and *Brassica Napus* which is used as an illuminant.

By drawing a current of air through the oil heated to 70° a so-called "blown" oil is produced, the specific gravity of which becomes almost equal to that of castor oil, namely 0·97; in this condition it is miscible with mineral oils. The mixture which is known as marine oil is used for lubricating marine engines.

LINSEED OIL is obtained by pressing the seeds of *Linum usitatissimum* either with or without the application of heat; the residues after compression are made up into cattle food.

The drying vegetable oils, particularly linseed oil, are used in the manufacture of oil paints as vehicles for the pigments; for artist's white paints, walnut and poppy-seed oils are chiefly used. The drying properties of linseed oil used for the manufacture of paint are greatly increased by boiling with lead oxide; such oil is known as boiled oil. A similar effect may be produced by dissolving in it certain salts known as "driers," such as lead linoleate or the metallic salts of resin acids, etc.

Varnish consists of a mixture of boiled oil with gum resins and oil of turpentine.

CASTOR OIL is obtained by compressing the seeds of *Ricinus communis* either with or without the application of heat. The seeds contain a fat-splitting enzyme which is employed commercially for the hydrolysis of fats; they also contain a very poisonous toxalbumin, known as Ricin, which remains in the residues after the expression of the oil. Castor oil is a thick viscid colourless liquid; when heated above 280°

it decomposes with the formation of oenanthol, a substance having a very unpleasant odour. Castor oil is largely used in the dye industry; for this purpose it is converted into the so-called turkey red oil, used for alizarin dyeing, by treatment with sulphuric acid and neutralization of the resulting sulphonic acid with soda.

For the manufacture of soap the following fats and oils are used: tallow fat, palm oil, palm-kernel oil, coconut oil and olive oil; the fats are boiled with caustic soda until saponification is complete, whereupon the mixture is saturated with common salt. The soap, being insoluble in strong salt solution, rises to the surface leaving the glycerol and salt in the aqueous layer below; the latter is then run off and the scum, which is allowed to harden in moulds, is known as hard soap. Soft soaps are prepared by boiling the cheaper oils, such as hemp-seed oil, cotton-seed oil or linseed oil with caustic potash; when saponification is complete the mixture is allowed to set to a semi-solid without the addition of sodium chloride; the resulting mixture contains all the glycerol together with the excess of alkali and a quantity of water.

The whole of the glycerol of commerce is obtained from fats; it is used largely for the manufacture of dynamite.

## CONSTITUTION OF FATS.

Fats belong to that class of organic compounds which are known as esters, an ester occupying in organic chemistry exactly the same position as salts do in inorganic chemistry.

When an inorganic base such as caustic soda or potash reacts with an acid, either organic or inorganic, one or more of the replaceable hydrogen atoms in the acid is replaced by the metal and the resulting product is known as a salt; thus:—

$$NaOH + H_2SO_4 = NaHSO_4 + H_2O$$

$$\text{or } KOH + CH_3COOH = CH_3COOK + H_2O$$

Acetic acid — Potassium acetate

If now the inorganic base be replaced by its organic analogue, an alcohol, a similar reaction ensues, but the resulting compound is called an *ethereal salt* or *ester*.

$$C_2H_5OH + H_2SO_4 = \underset{\text{Ethyl hydrogen sulphate}}{C_2H_5HSO_4} + H_2O$$

$$\underset{\text{Ethyl alcohol}}{C_2H_5OH} + \underset{\text{Acetic acid}}{CH_3COOH} = \underset{\text{Ethyl acetate}}{CH_3COOC_2H_5} + H_2O$$

The reaction between an acid and an alcohol containing three hydroxyl groups (OH) instead of only one, may be expressed by the following equation :—

$$\begin{array}{l} CH_2OH \\ | \\ CHOH \\ | \\ CH_2OH \\ \text{Glycerol} \end{array} + \begin{array}{l} C_{17}H_{35}COOH \\ \\ C_{17}H_{35}COOH \\ \\ C_{17}H_{35}COOH \\ \text{Stearic acid} \end{array} = \begin{array}{l} C_{17}H_{35}COOCH_2 \\ | \\ C_{17}H_{35}COOCH \\ | \\ C_{17}H_{35}COOCH_2 \\ \text{Tristearyl glycerol or tristearin} \end{array} + 3H_2O$$

the resulting compound tristearin also being an ester.

The naturally occurring fats are mixtures of similar esters of glycerol with other fatty acids such as palmitic $C_{15}H_{31}COOH$ or butyric $C_3H_7COOH$ acids, or with the unsaturated acid oleic acid $C_{17}H_{33}COOH$.

A wax, on the other hand, is an ester of a monohydric alcohol as illustrated by the equation :—

$$\underset{\text{Palmitic acid}}{C_{15}H_{31}COOH} + \underset{\text{Myricyl alcohol}}{C_{30}H_{61}OH} = \underset{\text{Myricyl palmitate}}{C_{15}H_{31}COOC_{30}H_{61}} + H_2O$$

myricyl palmitate being the chief constituent of beeswax.

Lapworth and Pearson * have shown that the glycerol in fats may be directly replaced by a higher polyhydric alcohol such as mannitol. This replacement may be brought about by distilling olein or stearin with mannitol under reduced pressure in the presence of sodium ethoxide. By this treatment much of the glycerol of the fat is expelled, the maximum yield being reached when the proportion of fat to the mannitol corresponds with two molecules of the former to three molecules of the latter. The composition of the mannitol olein, or mannitol stearin, corresponds with that of a mixture of dioleates or distearates of mannitan and isomannide. It has been shown by feeding experiments that mannitol olein is utilized by animals to the same extent as olive oil.†

* Lapworth and Pearson: "Biochem. Journ.," 1919, 13, 296.
† Halliburton, Drummond and Cannan: *id.*, 1919, 13, 301.

The classification and identification of fats is based upon the acids which they contain. Thus it is found that whereas beef suet and mutton fat consist chiefly of esters of the higher fatty acids, such as palmitic and stearic acids, butter contains a considerable quantity of the lower members of this same fatty series such, for example, as butyric, caproic, caprylic and capric acids; these acids, which are low boiling liquids readily volatile with steam, are known as volatile fatty acids and their presence or absence in a given sample of fat may be used for characterizing the fat. Thus, for example, the estimation of the amount of volatile fatty acid serves to distinguish genuine butter from its substitute margarine, which is relatively poor in volatile acids and contains chiefly higher fatty acids.

The more important members of the fatty acid series are given in the following list:—

| | | |
|---|---|---|
| $HCOOH$ | or $CH_2O_2$ | Formic acid* |
| $CH_3COOH$ | „ $C_2H_4O_2$ | Acetic acid |
| $C_2H_5COOH$ | „ $C_3H_6O_2$ | Propionic acid* |
| $C_3H_7COOH$ | „ $C_4H_8O_2$ | Butyric acid |
| $\begin{matrix}CH_3\\CH_3\end{matrix}\!>\!CH\,CH_2CH_2COOH$ | „ $C_6H_{12}O_2$ | Isobutyl acetic or caproic acid |
| $CH_3(CH_2)_6COOH$ | „ $C_8H_{16}O_2$ | Caprylic acid |
| $CH_3(CH_2)_8COOH$ | „ $C_{10}H_{20}O_2$ | Capric acid |
| $CH_3(CH_2)_{10}COOH$ | „ $C_{12}H_{24}O_2$ | Lauric acid |
| $CH_3(CH_2)_{12}COOH$ | „ $C_{14}H_{28}O_2$ | Myristic acid |
| $CH_3(CH_2)_{14}COOH$ | „ $C_{16}H_{32}O_2$ | Palmitic acid |
| $CH_3(CH_2)_{16}COOH$ | „ $C_{18}H_{36}O_2$ | Stearic acid |
| $CH_3(CH_2)_{18}COOH$ | „ $C_{20}H_{40}O_2$ | Arachidic acid |
| $CH_3(CH_2)_{20}COOH$ | „ $C_{22}H_{44}O_2$ | Behenic acid |

It should be noted that these acids all conform to the general formula for the fatty acids, $C_nH_{2n}O_2$, in which "n" may have any value, odd or even, but only those in which "n" is an even number are found to occur naturally in fats; the alleged occurrence in natural fats of acids with an uneven number of carbon atoms has in every case, so far recorded, been refuted on careful re-examination.

It appears probable, moreover, that all naturally occurring fatty acids have a straight and not a branched carbon chain, so that it is open to question whether the *iso*-butyl acetic acid which is said to have been found in fats was not, in reality, normal caproic acid of the formula $CH_3(CH_2)_4COOH$.

* These acids do not occur in fats.

Besides acids of the fatty series whose general formula is $C_nH_{2n}O_2$, acids belonging to several other series, poorer in hydrogen than the above, are found in fats. The simplest example of such a series of acids is furnished by the acids of the Oleic series, the members of which differ from the corresponding members of the fatty acid series in having two atoms of hydrogen less.

Some of the more important acids of this group are given below.

1. Acids of the OLEIC or ACRYLIC series.
General formula $C_nH_{2n-2}O_2$.

| | |
|---|---|
| $C_5H_8O_2$ | Tiglic acid |
| $C_{18}H_{34}O_2$ | Oleic acid |
| $C_{18}H_{34}O_2$ | Elaïdic acid |
| $C_{18}H_{34}O_2$ | Iso-oleic acid |
| $C_{22}H_{42}O_2$ | Erucic acid |
| $C_{22}H_{42}O_2$ | Brassidic acid |

The most widely distributed of these acids is undoubtedly oleic acid, which, in the form of its glyceride triolein,

$$\begin{array}{l} C_{17}H_{33}COOCH_2 \\ \quad\quad\quad\quad | \\ C_{17}H_{33}COOCH \\ \quad\quad\quad\quad | \\ C_{17}H_{33}COOCH_2 \end{array}$$

forms an important constituent of most vegetable and animal oils.

2. Acids of the LINOLIC series.
General formula $C_nH_{2n-4}O_2$.

(*a*) Open chain compounds, $C_{18}H_{32}O_2$ Linolic acid and its various isomers.
(*b*) Cyclic compounds, $C_{16}H_{28}O_2$ Hydnocarpic acid.
$C_{18}H_{32}O_2$ Chaulmoogric acid.

3. Acids of the LINOLENIC series.
General formula $C_nH_{2n-6}O_2$.

$C_{18}H_{30}O_2$ Linolenic acid and its isomers.

4. Acids of the CLUPANODONIC series.
General formula $C_nH_{2n-8}O_2$.

$C_{18}H_{28}O_2$ Clupanodonic acid.

5. Acids of the RICINOLEIC series.
General formula $C_nH_{2n-2}O_3$.

$C_{18}H_{34}O_3$ Ricinoleic acid and its isomers.

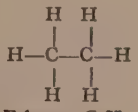
The relationship between the five series of acids, which differ from each other successively by two atoms of hydrogen, as shown by the formulæ—

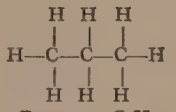
$$C_nH_{2n}O_2,\ C_nH_{2n-2}O_2,\ C_nH_{2n-4}O_2,\ C_nH_{2n-6}O_2,\ \text{and}\ C_nH_{2n-8}O_2$$

is similar to that subsisting between the three series of hydrocarbons having the general formulæ :—

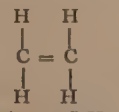
$$C_nH_{2n+2},\ C_nH_{2n},\ C_nH_{2n-2}$$

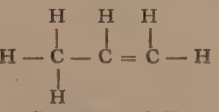
The hydrocarbons of the first or Paraffin series are said to be saturated, by which is meant that each of the four valencies of their carbon atoms are fully satisfied, as shown by the following graphic formulæ :—


Ethane $C_2H_6$


Propane $C_3H_8$

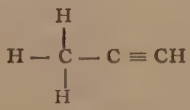
When, however, the graphic formulæ of the corresponding members of the second or Olefine series are written, it is found that if the tetravalency of carbon is maintained, there are not enough hydrogen atoms to satisfy all these valencies, and, in order not to leave any unsatisfied, the remaining valencies must be united to each other, thereby joining two carbon atoms to each other by more than one bond :—


Ethylene $C_2H_4$


Propylene $C_3H_8$

In the next series of hydrocarbons, the acetylenes, by the loss of two more hydrogen atoms, the process has been carried a step farther, with the result that two carbon atoms are united by a triple bond :—

Acetylene $C_2H_2$


Allylene $C_3H_4$

All such substances containing two carbon atoms united together by more than one bond are said to be unsaturated,

and are able to form additive compounds with many substances, notably the halogens.

Thus, while the saturated hydrocarbon will only react with chlorine or bromine by the replacement of one atom of hydrogen for each atom of halogen introduced into the molecule,

$$C_2H_6 + Br_2 = \underset{\text{Ethyl bromide}}{C_2H_5Br} + HBr$$

an unsaturated compound, such as ethylene, will add on the halogen directly—

$$C_2H_4 + Br_2 = \underset{\text{dibromide}}{\overset{\text{Ethylene}}{C_2H_4Br_2}}$$

the resulting additive compound being saturated.

It will thus be seen that it requires two atoms of bromine to saturate an unsaturated compound containing one double bond, and similarly it requires four atoms of halogen to saturate a compound containing two double bonds. In this way it is shown that since the acids of the oleic, linolic, and linolenic series require two, four, and six atoms of halogen respectively for saturation, they must contain respectively one, two, or three double bonds.

A measure of the degree of unsaturation of a given acid may accordingly be obtained by determining how much bromine it will absorb; as, however, the interaction with bromine is liable to be violent it is found more convenient to employ iodine, which, in addition to being less violent in its action than bromine, is also easier to handle.

A description of the method employed in determining what is known as the "iodine value" of fats is given below (p. 31).

## EXTRACTION OF FATS.

The isolation of fats from admixture with other substances may be effected by extraction by means of fat solvents.

The principle of the extraction is to treat the dried mixture with a solvent which will dissolve only the fat and leave the other substances unchanged. The solvents most commonly used for this purpose are ether, light petroleum, carbon tetrachloride and carbon disulphide, the two latter being used chiefly on a commercial scale.

It must be borne in mind that besides extracting fats, ether will also dissolve essential oils, cholesterol, lecithin and allied substances variously known as lipoids, lipins,* etc.

Moreover, other substances which are of themselves insoluble in ether may become soluble in the presence of fats.

Whatever solvent is employed must be tested before use to see that it leaves no residue on evaporation and is free from moisture.

A rough and ready method of extracting fat from a given sample is to place the finely divided and dried material on a filter paper folded into a funnel and to pour the fat-solvent on to it. The filtrate will contain most of the fat which may be recovered by evaporating off the solvent.

When it is desired to extract the fat quantitatively, the operation is most conveniently carried out in a Soxhlet apparatus (see below).

Previous to extraction, the substance must be thoroughly dried. For this purpose it must either be gently heated in a current of dry air or else desiccated by means of alcohol or anhydrous salts.

The first method, which is the most convenient, should, however, be used with caution, as many fats may undergo chemical change during the process, as a result of which the material extracted by ether after drying may be very different from the substance originally present in the moist sample.

The second method, which consists in treating the sample to be dried with absolute alcohol for some hours and then filtering and pressing, depends on the fact that the alcohol withdraws the water without dissolving away any appreciable quantity of the fat; if treated two or three times in this way the substance will be practically free from moisture and can then be extracted under a Soxhlet with ether. The wet alcoholic filtrates on careful evaporation yield a residue which may be separately treated with ether to extract any fat contained in them. It is unnecessary to remark that the method cannot be employed if the fat to be extracted is soluble in alcohol.

The third method of drying, which involves the use of an-

* For an account of this group of substances see Maclean: "Lecithin and Allied Substances. The Lipins," London, 1918.

hydrous salts such as sodium sulphate, depends on the fact that the anhydrous salt when ground up with the moist tissue withdraws the water from it, forming the hydrated crystals. In a few hours the substance is sufficiently dry to be powdered. The chief objection to this process is the fact that a considerable bulk of salt has to be employed and consequently the volume of the material to be extracted is much increased.

### PHYSICAL PROPERTIES OF FATS.

The naturally occurring fats vary in consistency from oils to wax-like solids; the solid fats have mostly a low melting point which is, however, rarely a sharp one, as natural fats are not simple substances, but are, as a rule, mixtures of several different chemical individuals; such mixtures never have sharp melting points.

All fats and fatty oils are lighter than water, their specific gravity varying from about 0·900 to 0·970 at 15°. They are insoluble in water and at ordinary temperatures are sparingly soluble in cold alcohol, excepting castor oil which dissolves readily in this solvent; they, however, dissolve readily in ether, chloroform, petroleum ether, benzene, carbon tetrachloride or carbon disulphide.

### CHEMICAL PROPERTIES OF FATS.

One of the most important chemical properties of fats is their decomposition by hydrolysis.

The term hydrolysis, which literally means loosening by water, is applied to any reaction in which a substance is broken up into two or more simpler ones with the fixation of water.

The following examples taken from a variety of different classes of compounds all illustrate this reaction:—

$$\underset{\text{Ethyl acetate}}{CH_3COOC_2H_5} + H_2O = \underset{\text{Acetic acid}}{CH_3COOH} + \underset{\text{Ethyl alcohol}}{C_2H_5OH} \quad (1)$$

$$\underset{\text{Methyl cyanide}}{CH_3CN} + 2H_2O = CH_3COOH + NH_3 \quad (2)$$

$$\underset{\text{Hippuric acid}}{C_6H_5CONHCH_2COOH} + H_2O = \underset{\text{Benzoic acid}}{C_6H_5COOH} + \underset{\text{Glycine}}{NH_2CH_2COOH} \quad (3)$$

$$\underset{\text{Malt sugar}}{C_{12}H_{22}O_{11}} + H_2O = \underset{\text{Glucose}}{2C_6H_{12}O_6} \quad (4)$$

$$\underset{\text{Amygdalin}}{C_{20}H_{27}NO_{11}} + 2H_2O = \underset{\text{Benzaldehyde}}{C_6H_5CHO} + \underset{\text{Glucose}}{2C_6H_{12}O_6} + \underset{\text{Hydrocyanic acid}}{HCN} \quad (5)$$

It will be seen from reaction (1) that the conversion of an ester into an acid and an alcohol is an example of hydrolysis, and since fats are esters it follows that they also must be capable of hydrolysis.

The reaction—

$$\begin{array}{l} C_{17}H_{35}COOCH_2 \\ C_{17}H_{35}COOCH \\ C_{17}H_{35}COOCH_2 \end{array} + 3H_2O = 3C_{17}H_{35}COOH + \begin{array}{l} CH_2OH \\ CHOH \\ CH_2OH \end{array}$$

Stearic acid — Glycerol

is, however, not readily brought about by water alone at ordinary temperatures ; in the presence of enzymes, however, the hydrolysis may be effected at a moderate temperature with comparative ease (see p. 370).

The hydrolysis of fats for the purpose of preparing the *free fatty acids* may be effected in either of the following ways :—

1. By acting on the fat with superheated steam in the presence of a little lime or magnesia, which acts as a catalytic agent.

This method is the one most commonly adopted by candle-makers for the preparation of fatty acids required in the manufacture of candles. The fat is subjected to the action of steam under pressure at 170° in large copper vessels in the presence of a small quantity of lime. The resulting mixture is then treated with sulphuric acid sufficient in amount to combine with the lime, after which the free fatty acids rise to the surface in a molten condition.

2. By the action of concentrated sulphuric acid.

The molten fats are stirred up in leaden vessels with 9 per cent of concentrated sulphuric acid, the mixture being heated to about 120° C. The mixture is then warmed with water to remove the acid, and the acids are further purified by distillation with steam.

### SAPONIFICATION OF FATS.

Closely related to hydrolysis is the reaction known as saponification ; this reaction, which literally means "soap-making," is that which takes place when a fat is boiled with caustic alkali. The alkali acts in much the same way as water, breaking up the ester into glycerol and the fatty acid

which, however, in this case, combines with the alkali to form a salt :—

$$\begin{array}{l} C_{17}H_{35}COOCH_2 \\ | \\ C_{17}H_{35}COOCH \\ | \\ C_{17}H_{35}COOCH_2 \\ \text{Tristearin, a fat} \end{array} + 3KOH = \underset{\text{Potassium stearate, a soap}}{3C_{17}H_{35}COOK} + \begin{array}{l} CH_2OH \\ | \\ CHOH \\ | \\ CH_2OH \\ \text{Glycerol} \end{array}$$

It so happens that the sodium and potassium salts of palmitic, stearic, and oleic acids dissolve in water forming opalescent alkaline solutions which readily give a lather, and can, therefore, be used as soaps,* and hence the process by which they are made from fats is called saponification. Although alkali metal salts of other organic acids do not exhibit the characteristics of soap, the term saponification has nevertheless been extended to include all cases of the decomposition of an ester into the corresponding alcohol and the salt of the acid,

* The sodium and potassium salts of oleic acid and of the higher fatty acids, such as palmitic and stearic acids, when dissolved in water, are, to a large extent, hydrolysed into free fatty acid and caustic soda according to the equation :—

$$\underset{\text{Sodium stearate}}{C_{17}H_{35}COONa} + H_2O = \underset{\text{Stearic acid}}{C_{17}H_{35}COOH} + NaOH$$

The stearic acid combines with some of the unhydrolysed soap to form an insoluble acid salt, giving rise to an opalescent or turbid solution. It is this insoluble acid salt which is responsible for the formation of a lather on shaking such a solution. The detergent or cleansing action of soap is dependent on the above reaction since the caustic soda detaches the greasy dirt which then becomes enveloped in a layer of soap solution from the lather and is so carried away.

In this connexion it is interesting to note the similar effect of soap on the formation of emulsions.

An emulsion may be defined as a mixture, under special conditions, of two otherwise immiscible liquids. Thus, for example, if olive oil is shaken up with water, the two liquids rapidly separate as soon as the shaking ceases. If, however, a little soap solution or some other substance such as gum acacia, tragacanth, saponin (see p. 181), or white of egg be added and the shaking repeated, an emulsion results owing to the oil particles being enveloped in a layer of soap or other substance which prevents their coalescing. Milk is an example of a naturally occurring emulsion; so also is latex contained in plants.

If pure olive oil, free from oleic or other acid, is shaken up with caustic soda no emulsion is produced; on the other hand, olive oil which has been kept some time and contains free oleic acid, when shaken up with caustic soda does produce an emulsion, thus showing that the emulsifying agent is not the free alkali but the soap produced in the second case from the soda and the oleic acid.

This may be also illustrated by Bütschli's experiment which consists in placing a drop of old olive oil containing 9 per cent of oleic acid on a little 0·06 per cent aqueous solution of sodium carbonate. If examined under the microscope it will be seen to consist of a fine honeycomb structure, consisting of particles of oil, the whole apparently exhibiting amœboid movements; these latter are due to difference in surface tension.

even though that salt may have none of the characteristic properties of a soap.

The saponification of a fat on a small scale * in the laboratory may be effected as follows: boil the fat with about three or four times its weight of alcoholic potash under a reflux condenser. The alcoholic potash is prepared by dissolving caustic potash in about twice its weight of water and mixing the solution with twice its volume of alcohol. The heating should be continued until on pouring a little of the solution into a large volume of water an opalescent solution free from undecomposed fat results. The time required for this may vary from a few minutes to half an hour or more.

When the saponification is complete, the contents of the flask should be heated in an evaporating basin over a water bath, and thoroughly stirred to get rid of the alcohol. If the free fatty acids are required sufficient sulphuric acid is then added to make the solution strongly acid, whereupon the fatty acids separate out and rise to the surface.

The aqueous layer contains the glycerol together with the excess of sulphuric acid and potassium sulphate.

### CHOLESTEROL AND PHYTOSTEROL.

In addition to the trihydric alcohol glycerol, all fats contain a small quantity of the monohydric alcohols cholesterol and phytosterol † which form what is known as the *unsaponifiable* residue of fats.

These substances may be isolated from fats according to the following method devised by Kossel and Obermüller.‡

An ethereal solution of the fat is mixed with a solution of sodium in alcohol; saponification takes place in the cold and the soap which is precipitated from solution can be filtered off; the filtrate, which is a mixture of alcohol and ether, contains the glycerol together with the so-called unsaponifiable residue consisting of phytosterol or cholesterol which may be obtained by evaporating the solvent.

* For commercial soap manufacture, see p. 5.

† The term phytosterol though employed by many authors to indicate a single definite substance is beginning to be used as a generic term for a whole group of closely allied substances the number of which is rapidly increasing as the investigation of vegetable fats proceeds.

‡ Kossel and Obermüller: "Zeit. physiol. Chem.," 1890, 14, 599; 1891, 15, 321.

The following method originally due to Allen and Thomson is recommended by Lewkowitsch for the estimation of the "Unsaponifiable Residue".

Five grams of the fat or oil are saponified by boiling under a reflux condenser with 25 c.c. of alcoholic potash containing 11·2 per cent of caustic potash; when saponification is complete the alcohol is evaporated off and the residual soap is dissolved in 50 c.c. of hot water and transferred to a separating funnel of about 200 c.c. capacity, about 20-30 c.c. of water being used to rinse out the dish. After cooling, the mixture is shaken with 30-50 c.c. of ether and set aside until the ethereal layer has separated. (*N.B.*—The separation is accelerated by the addition of a little alcohol.) The soap solution is then run off from below into a second separating funnel and shaken once more with a fresh quantity of ether. Two extractions should suffice, but it is safer to extract a third time. The ethereal extracts are then united, washed with a small quantity of water to remove any soap and transferred to a weighed flask; after evaporating off the ether, the flask is weighed again; the increase in weight gives the amount of unsaponifiable residue in 5 grams of the sample.

The isolation and identification of the unsaponifiable residue is of considerable importance for the purpose of establishing whether a given sample of fat or oil is of animal or vegetable origin, since animal fats all contain cholesterol while vegetable fats contain either phytosterol itself or a closely allied substance belonging to the group of phytosterols.

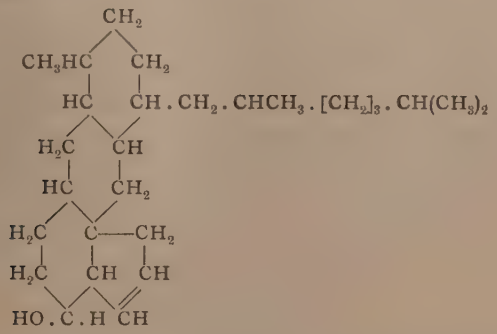
## REACTIONS AND PROPERTIES OF CHOLESTEROL AND PHYTOSTEROL.

### *Cholesterol.*

Cholesterol is a monohydric alcohol of the formula $C_{27}H_{45}OH$; its constitution is still unknown, although a great deal of work has been expended on this question. According to Windaus * it would appear to be a secondary

* Windaus: "Nachr. K. Ges. Wiss. Göttingen," 1919, 237; see also Dorée: "Biochem. Journ.," 1909, 4, 72.

alcohol containing an unsaturated group as expressed by the formula :—




Cholesterol occurs in the bile, certain gall stones, brain, blood and wool fat. It is insoluble in water and crystallizes from chloroform in needles and from ether or alcohol in rhombic plates, m.p. 148-150°. It may conveniently be obtained by evaporating the ethereal extract of gall stones to dryness.

*Reactions.*—(1) Crystals of cholesterol pressed on a white porcelain surface and moistened with a drop of sulphuric acid (5 parts concentrated acid to 1 part of water) turn pink. The addition of a drop of dilute iodine causes a play of colours from red to blue or green.

(2) A solution of cholesterol in chloroform gently agitated with concentrated sulphuric acid turns red, while the sulphuric acid which forms the lower layer assumes a green fluorescence.

(3) On the addition of concentrated sulphuric acid drop by drop to a little cholesterol dissolved in a mixture of 2-3 drops of chloroform and about 10 drops of acetic anhydride, a transient pink colour is at first formed; on the addition of more acid, however, the colour changes to blue and finally to green.

(4) Alcoholic solutions of cholesterol mixed with a few drops of 1 per cent alcoholic solution of digitonin,* give an immediate white precipitate, $C_{27}H_{46}OC_{54}H_{92}O_{28}$, a reaction employed in the estimation of cholesterol.†

* Panzer: "Chem. Zentr.," 1912 (ii.), 540.

† Windaus: "Ber. deut. chem. Gesells.," 1909, **42**, 238; "Z. physiol. Chem.," 1910, **65**, 110; Salomon: "Ber. deut. pharm. Gesells.," 1914, **24**, 189.

*Phytosterols.*

The term phytosterol was at one time employed to designate a definite chemical individual of the formula $C_{27}H_{45}OH$, but it is now used more as a generic term to include a number of different substances having certain properties in common. Thus Windaus and Hauth* showed that the substance obtained from Calabar beans and commonly known as phytosterol was in reality a mixture of two substances—(*a*) Sitosterol of the formula $C_{27}H_{45}OH$, and (*b*) Stigmasterol $C_{30}H_{47}OH$, an observation which has been confirmed by Salway.†

Similarly Klobb ‡ describes a dextro-rotatory phytosterol of the formula $C_{31}H_{52}O$, $3H_2O$ occurring in *Anthemis nobilis* and a number of lævo-rotatory phytosterols of different formulæ obtained from *Matricaria Chamomilla*, *Tilia europaea*, *Linaria vulgaris*, and *Verbascum Thapsus.*§

All vegetable fats contain phytosterol, the amount varying from about 0·13 to 0·30 per cent and rising in the case of pea fat and the fat of Calabar beans to a considerably higher value. In the case of the wheat, the grain of which contains sitosterol whilst the bran contains a different phytosterol, the amounts of these substances differ in the various parts of the plant. The percentage present in the green parts is higher than the percentage occurring in the grain which is somewhat greater as compared with the percentage in etiolated plants. The fact that the highest percentage occurs in the embryo suggests a function in connexion with germination and growth; not necessarily a direct nutritive function since a starved plant contains as much as the grain. ‖

The phytosterols are widely distributed in the vegetable kingdom: in addition to the higher plants, they occur in *Sphagnum*, *Laminaria*, *Agaricus*, *Lactarius*, and *Polyporus.* ‖

* Windaus and Hauth: "Ber. deut. chem. Gesells.," 1906, **39**, 4378; 1907, **40**, 3681.

† Salway: "J. Chem. Soc.," 1911, **99**, 2154.

‡ Klobb: "Compt. rend.," 1911, **152**, 327; "Ann. Chim. Phys.," 1911, viii., **24**, 410.

§ See also Power and Rogerson: "J. Chem. Soc.," 1910, **97**, 1951; Rogerson: "Amer. Journ. Pharm.," 1911, **83**, 59; "J. Chem. Soc.," 1912, **101**, 1040.

‖ Ellis: "Biochem. Journ.," 1918, **12**, 154, 160, 173.

Phytosterol crystallizes from alcohol in elongated plates and from ether in slender needles. The melting point of the pure substance varies somewhat according to the source from which it is prepared; it lies somewhere between 135 and 137° or it may be as high as 144°. The reason for this may be that the various substances obtained from different sources and described as one and the same substance are in reality different substances but all of a phytosterol nature. The colour reactions of phytosterol resemble those of cholesterol.

Cholesterol and phytosterol cannot with certainty be distinguished by means of their melting points, owing to the fact that phytosterol may melt at any temperature between 135 and 144° according to the source from which it is prepared. As, however, there is a considerable difference between the melting points of the acetates of these two substances the following procedure is recommended by Lewkowitsch.

The unsaponifiable residue remaining after evaporation of the ether (p. 15) is dried over a water bath and then dissolved in the least possible quantity of absolute alcohol and allowed to crystallize. The crystals which separate should be examined under a microscope; cholesterol crystallizes in four-sided plates and phytosterol in elongated hexagonal plates.

The alcohol is then evaporated off completely and the residue is carefully heated with 2 to 3 c.c. of acetic anhydride over a free flame until the liquid boils, the remaining acetic anhydride being evaporated off over a water bath. The residue is then re-crystallized two or three times from the least possible quantity of absolute alcohol, and the melting point of the crystals so obtained is determined.

Cholesterol acetate melts at 114·3-114·8°.

Phytosterol acetate * melts at 125-137°.

## SPONTANEOUS CHANGES IN FATS.

*Rancidity.*—Most fats when exposed to air and light sooner or later become rancid, acquiring an unpleasant taste and smell. The actual cause of this change is as yet but little understood,

* The acetyl derivative obtained by Power and Moore from the root of *Bryonia* has the melting point 155-157°.

though it appears probable that it is the result of the combined action of a number of different factors such as oxygen, light, moisture, bacteria and enzymes; the complex fats, and possibly also the small quantities of proteins and other impurities contained in them, are thereby broken down into simpler bodies such as the lower volatile fatty acids and aldehydes. It is frequently true that a considerable quantity of free acid is liberated in fats which have become rancid, and this is especially so in the case of fats such as butter which contain acids of low molecular weight, as butyric acid, the smell of which recalls that of rancid butter. It is, however, a fact that a fat may be acid without being rancid* cocoa butter, for instance, has usually an acid reaction but very rarely becomes rancid.

With regard to other constituents found in rancid fats, various authors have from time to time observed the presence of hydroxy-acids, aldehydes, alcohols, and of esters of lower fatty acids, but there appears to be a general consensus of opinion that glycerine does not occur.

*Drying and Resinification.*—Most fatty oils on exposure to the air tend to thicken, owing partly to polymerization and partly to oxidation; in some cases the oil actually dries up, leaving a more or less hard mass or a thin elastic film.

Those oils which only thicken, without actually becoming hard or dry, are called *non-drying* oils. They are composed for the most part of triolein (cf. p. 8) and contain only small quantities of solid fatty acids; to this class of oils belong the following: olive oil, almond oil, arachis or pea-nut oil, quince oil, cherry- plum- peach- and apricot-kernel oil, wheat-meal oil, rice, tea-seed oil, and hazel-nut oil.

Two further oils, namely castor oil and grape-seed oil, are also included in this group of non-drying oils, but they have a slightly different composition from the other members of this group. They are characterized by possessing a considerable percentage of glycerides of hydroxylated fatty acids, such as dihydroxystearic acid, a fact which is brought out clearly by their high acetyl values (p. 35).

In contrast with these non-drying oils are the so-called *drying oils*, among the more important of which are the follow-

* Vintilesco and Popesco: "J. Pharm. Chim.," 1915 [iv.], **12**, 318.

ing: linseed oil, cedar-nut oil, hempseed, walnut, poppy-seed, and sunflower oil. These oils exhibit to a greater or less degree the tendency to absorb oxygen from the air, thereby drying up and leaving an elastic skin, a property which is made use of industrially in the manufacture of oil paints. These drying oils are composed chiefly of the glycerides of the unsaturated acids of linolic and linolenic series and contain only relatively small quantities of oleic acid. Owing to the large amount of unsaturated acids which they contain their iodine value (p. 30) is very high (120–200).

In addition to the above there is also a third group of vegetable oils known as the *semi-drying* oils whose iodine value and drying properties lie midway between those of the drying and non-drying oils. They differ from the true drying oils in containing no acids of the linolenic series and from the non-drying oils in containing linolic acid. The oils belonging to this category fall naturally into two sub-groups:—

(1) The cotton-seed oil group, to which belong Soja-bean oil, maize oil, pumpkin, water-melon and melon-seed oils, beech-nut oil, cotton-seed, sesame and croton oils, and the lesser known oils of the apple, pear, orange, barley and rye seeds.

(2) The rape oil group comprising garden cress, hedge mustard, wild radish, black mustard seed, white mustard seed, radish seed and rape or colza oil.

The oils of the latter sub-group have a lower saponification value (p. 30) than any other vegetable oils, and arachidic acid seems to be a normal constituent of them all.

To determine whether an oil is a drying one or not, a drop is spread on a glass plate, such as a microscope slip, and left for several days at atmospheric temperature. Non-drying oils such as olive and castor oils are unaltered after about eighteen days: semi-drying oils such as cotton-seed, sesame and rape oil are more or less dry, but still sticky in from seven to eight days, whereas real drying oils like poppy and especially linseed are quite dry in from three to six days.

### GENERAL PROPERTIES AND REACTIONS OF FATS.

(1) All fats, both solid and liquid, are soluble in ether, light petroleum, carbon tetrachloride, chloroform and carbon di-

sulphide, but are only sparingly soluble in alcohol and insoluble in water.

(2) Being esters of glycerol they all contain this substance as may be proved by heating any fat with potassium hydrogen sulphate, whereby the glycerol is broken down into acrolein, which may be detected by its unpleasant odour.

$$CH_2OHCHOHCH_2OH = CH_2:CHCHO + 2H_2O$$

In order to show that the hydrolysis of fats gives rise to glycerol some fat should be saponified as described above and then acidified with hydrochloric acid; after filtering off the fatty acids, the filtrate is evaporated to small bulk over a water bath and the residue is extracted with alcohol, which dissolves out the glycerol leaving behind the salts. The presence of glycerol in the alcoholic extract may be proved by evaporating to dryness and applying the following test: two drops of the residue are carefully heated to about 120° with two drops of molten phenol and an equal quantity of concentrated sulphuric acid; the resulting resinous mass on cooling gives a brown solid which dissolves in ammonia forming a carmine-coloured solution.

(3) All fats can be saponified by boiling with alcoholic potash. For this purpose 2 grams of fat may be boiled for fifteen minutes with 25 c.c. of 3 per cent alcoholic potash. The resulting mixture of potassium soap and glycerol is soluble in water; on acidifying the solution the free fatty acids are precipitated.

(4) Fats leave a translucent mark on paper. Similar stains may be left by substances other than fats, but in most cases the stains disappear fairly rapidly as the substance evaporates.

### SPECIAL TESTS FOR PARTICULAR CLASSES OF FATS.

*Elaïdin Test.*—This test, which is distinctive of the oleic series, depends on the fact that nitrous acid converts liquid olein into solid elaïdin, while it has no corresponding action on glycerides of linolic, linolenic, or isolinolenic acids. The test may be performed as follows:—

Ten grams of oil are shaken in a test tube with 5 grams of nitric acid (sp. gr. 1·38-1·41) and 1 gram of mercury for

three minutes or more until the mercury is completely dissolved. After the lapse of twenty minutes, the mixture is shaken for another minute, and is then set aside and the time noted which is required for the oil to solidify.

Olive oil requires one hour.

Arachis or earth-nut oil requires one and a half hours.

Colza and sesame oil require three hours.

Linseed oil gives a red pasty froth.

Hempseed oil remains unchanged.

The temperature of the mixture must be maintained constant during the test, and must not exceed 25°.

*Bromide Test.*—This test, which is chiefly used for distinguishing between drying and semi-drying oils, depends on the fact that linolic, linolenic acids and other unsaturated acids produce insoluble additive compounds with bromine, containing six or eight atoms of this element. According to Hehner and Mitchell* from 1-2 c.c. of oil are dissolved in 40 c.c. of ether containing a few cubic centimetres of glacial acetic acid; the mixture is then cooled to 5° and treated with bromine drop by drop until no more is absorbed. After three hours the precipitate is filtered off on a tared asbestos filter and washed four times with 10 c.c. of ether; it is then dried in a steam oven. The weight of the precipitate is directly proportional to the amount of unsaturated acids present in the fat.

*Sulphuric Acid Test.*—On mixing fats of the oleic series with concentrated sulphuric acid no heat is evolved, whilst with fats of the linolic series the opposite is the case.

### COLOUR REACTIONS OF INDIVIDUAL FATS.

Many of the colour reactions described for fats are of doubtful value owing to the modifying influence of small quantities of resins and of proteins. The following tests are, however, fairly reliable:—

*Badouin's Test for Sesame Oil.*—Twenty c.c. of sesame oil are thoroughly shaken for a short time with 10 c.c. of hydrochloric acid (sp. gr. 1·9) containing 0·18 gram of cane sugar. A rose colour should appear immediately after the two layers of oil and water have separated; if left to stand longer the sugar solution causes a brown coloration.

* Hehner and Mitchell: "The Analyst," 1898, **23**, 313.

*Solstien's Reaction for Sesame Oil.*—Two or three volumes of oil or fat are dissolved in twice their volume of benzene (b.p. 70-80°) and gently shaken with three volumes of concentrated zinc chloride saturated with hydrochloric acid, the whole being kept immersed in a water bath at a temperature of 40° C.; when the zinc chloride has sunk to the bottom, the test tube is immersed up to the top level of the zinc chloride in a water bath at 80° C. If sesame oil is present a pink colour is produced.

*Halphen's Reaction for Cotton-seed Oil.*—Equal volumes of oil, amyl alcohol, and a one per cent solution of sulphur in carbon bisulphide are mixed in a test tube immersed to half its depth in boiling brine for about ten minutes. By the end of this time an orange colour should appear; if not add more carbon bisulphide and boil again. The colour is said to be due to the addition of sulphur to an unsaturated bond.

*Sulphuric Acid Test.*—Rape-seed oil and mustard oil when shaken with sulphuric acid (sp. gr. 1·53-1·62) produce grass-green to blue-green colours. Linseed and hemp oil may also give similar colorations.

### MICROCHEMICAL REACTIONS.

1. The microscopical appearance of oil when mixed with water is characteristic owing to its immiscibility with water and its different refractive index.

2. Its solubility in ether, chloroform, benzene, or other fat solvents is easily noted.

3. If oil be present in the preparation it will fairly rapidly turn brown and then black when treated with a one per cent solution of osmic acid. This is not absolutely conclusive since osmic acid stains proteins brown.

4. Tincture of alkannin, or a saturated solution of Scharlach R in 75 per cent alcohol, colours oil globules red or pink.

The reaction with the first-named reagent is often ill-defined and frequently fails when the alkanna used has been extracted from the root some time. The test is more satisfactory when freshly prepared tincture is used.

A similar reaction is given by Sudan III.

It is important to note that these and similar reactions are not conclusive of the chemical nature of the substances acted

upon. For example, Sudan III not only stains oils red but also resins, latex, wax, and cuticle; chloroplasts are stained a pale red; cellulose, lignified walls, gelatinized membranes, starch, and tannin are unstained.

The staining tests mentioned above may be employed after extracting the oil with ether or other solvent.

### QUANTITATIVE ESTIMATION OF FATS.

1. *By Means of Soxhlet's Extraction Apparatus.*—The fact that oils and fats are readily dissolved by ether, chloroform, and light petroleum, is made use of in their estimation; but it must be borne in mind that the method only yields correct results provided other substances, which would be extracted by the solvent employed, are absent from the material under examination.

The general arrangement of the apparatus required is given in Fig. 1. The flask F, which is half-filled with the solvent to be employed, is connected to the extractor by a closely fitting cork. The material to be extracted is put into a thimble made of special quality filter paper and placed in the extractor, which is connected to a reflux condenser (C).

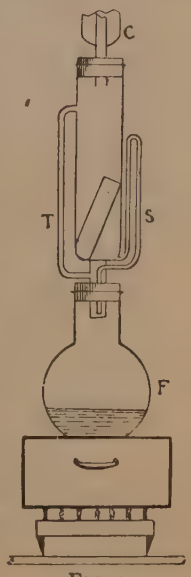


FIG. 1.

The method may be conveniently employed for determining roughly the proportion of oil in the reserve food of the castor-oil seed, for example.

A number of seeds, freed from their testas, are carefully weighed and one by one are broken up in a perfectly clean glass basin with the well-rounded end of a glass rod. The material is then dropped into the thimble; any particles adhering to the basin or rod must be carefully removed by means of a platinum wire and also placed in the thimble.

The basin and glass rod should then be carefully washed with a few drops of ether to remove the last traces of fat, and the resulting solution should be added to the broken-up seeds in the thimble, care being taken not to employ enough ether for the solution to trickle through the thimble.

The thimble is suspended in a steam oven for half an hour, and is then placed inside the extractor; a few small chips of porcelain are placed in the flask F, and the whole carefully weighed and then half-filled with freshly distilled ether. The apparatus is then connected up. The ether in the flask F volatilizes and passes up the tube T into the extractor and condenser, and gradually fills the Soxhlet; on reaching a certain level it siphons over into the flask, carrying with it the fat in solution; once in the flask the ether is again vaporized and goes through the same process as before, the oil, however, remains behind. The ether is allowed to siphon off at least a dozen times,* and then, when most of the ether is in the extractor, the flask is disconnected. The ether in the flask is evaporated off and the flask is placed in a steam oven for half an hour, it is then allowed to cool in a desiccator and finally weighed.

| | |
|---|---|
| Weight of seeds | $x$ |
| Weight of flask, chips and oil | $y$ |
| Weight of flask and chips | $z$ |
| Weight of oil | $y-z$ |

$$\text{Per cent fat} = \frac{100\,(y-z)}{x}$$

If the ether has extracted substances other than fats, the result obtained will, of course, be too high. In such cases the ether extract may be saponified and the amount of fatty acid may be determined, from which the amount of fat originally present may be estimated.

2. *By Saponification.*—Apart from the fact that in some cases it is not possible to extract the fat quantitatively by Soxhlet's method with less than forty-eight hours' continuous extraction, the method is open to the objection that the substance must be dried previous to extraction, and this may involve loss or alteration of the fat; furthermore, the residue which is weighed as fat may not consist entirely of fat but may contain other substances which are extracted by the same solvents as the fats; this consequently necessitates a further examination of the residue for its saponification value, etc.

* The number of times the liquid should be allowed to siphon off varies in every case. In order to ensure complete extraction the only safe method to adopt is to weigh the fat extracted after a certain time, then to attach the flask again and continue the extraction for some time longer and again weigh.

The following method which is due to Liebermann and Székely * has the advantage of giving in a short time a reliable value for the percentage of fat in almost any substance, and is specially convenient for the estimation of fat in fodder, meat, fæces, and physiological work in general. Five grams of the sample are placed in a flask (of the dimensions given in Fig. 2) with 30 c.c. of 50 per cent caustic potash (sp. gr. 1·54). The mixture is boiled over a wire gauge for half an hour and frequently shaken. After cooling 30 c.c. of 90-94 per cent alcohol are added and the heating is continued for another ten minutes; the mixture is then cooled again and carefully mixed with 100 c.c. of 20 per cent sulphuric acid (sp. gr. 1·145) and thoroughly shaken after each addition; the temperature must be kept low so as to avoid any loss of volatile fatty acids. When quite cold 50 c.c. of light petroleum (sp. gr. 0·6-0·7; b.p. about 60° C.) are added, and the flask is then closed with a tightly fitting rubber stopper and is thoroughly shaken for about ten seconds; the shaking is repeated about thirty times at intervals of one or two minutes without removing the stopper. Saturated salt solution is then added until the lower aqueous layer reaches up to the 240 c.c. graduation which is marked on the neck. After shaking again a few times the flask is set aside in a vessel of cold water. When the petroleum containing the fatty acids in solution has separated, 20 c.c. are withdrawn by means of a pipette and are placed in a wide-mouthed 150 c.c. flask; 40 c.c. of 96 per cent alcohol, free from acid, are now added, together with 1 c.c. of a solution of phenolphthalein (made by dissolving 1 gram of accurately weighed phenolphthalein in 100 c.c. of 96 per cent alcohol) and the solution is titrated with N/10 alcoholic potash.

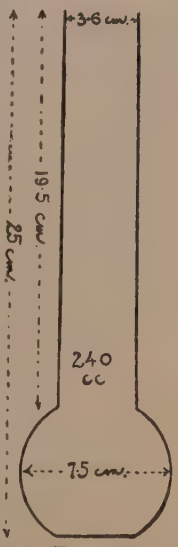


Fig. 2.

The titrated liquid is then carefully transferred in small portions at a time to a tared weighing bottle of about 80 c.c.

* Liebermann and Székely: "Plüger's Archiv," 1898, **72**, 360.

capacity, which is warmed over a gently boiling water bath; when the whole liquid has been evaporated to dryness, the residue is heated in an air oven for an hour at 100°, and, after cooling in a desiccator, is weighed with the glass stopper inserted to prevent the hygroscopic soap from absorbing any moisture from the air.

The amount of fat which corresponds to a given weight of soap may be calculated as follows:—

$$\begin{array}{lcl} C_{17}H_{35}COOK & & CH_2 \quad C_{17}H_{35}COOCH_2 \\ C_{17}H_{35}COOK - 3\,K + & & CH = C_{17}H_{35}COOCH \\ C_{17}H_{35}COOK & & CH_2 \quad C_{17}H_{35}COOCH_2 \\ \text{Soap} & & \text{Fat} \end{array}$$

From the above equation it will be seen that in order to convert three molecules of soap into one molecule of fat three atoms of potassium, $3 \times 39{\cdot}1$, have to be withdrawn from three molecules of soap, and have to be replaced by 41 parts of $CH_2 \cdot CH \cdot CH_2$; this is equivalent to deducting 39·1 from one molecule of soap and adding $\frac{41}{3}$ or 13·6, or, in other words, deducting 25·5.

Hence, if "n" is the number of centimetres of N/10 caustic potash required for the titration, and since 1 c.c. N/10 KOH ≡ ·00391 gram K ≡ ·00136 gram $C_3H_5$, we have to deduct from the weight of the soap $W_s$

$$n \times {\cdot}00391 \text{ and add } n \times {\cdot}00136$$

which is equivalent to deducting $n \times {\cdot}00255$.

Also, since 1 c.c. of phenolphthalein solution on evaporation would leave 0·01 gram of solid, this quantity must be deducted from the weight of the soap.

Hence the percentage of fat may be calculated from the relation

$$F = \left\{ \frac{W_s - {\cdot}01 - (n \times {\cdot}00255)}{m} \right\} \times 250$$

in which "m" is the weight of the sample taken.

In estimating fat in flour or farinaceous grain by this method, it is best to subject the substance to a preliminary treatment by heating 5 grams of the sample for half an hour with 30 c.c. of dilute sulphuric acid (1 : 10), the mixture is then diluted with 50 c.c. of 50 per cent. caustic potash. Finally

the liquid is acidified with 60 c.c. of sulphuric acid (sp. gr. 1·3) as described above. After the shaking with light petroleum is completed, 50 c.c. of 94 per cent alcohol are added instead of the salt solution; this has the effect of accelerating the separation of the petroleum layer which otherwise might take a long time.

Owing to the relatively small solubility of stearic acid in light petroleum the method may give too low a result in the case of substances very rich in stearin; the result should, therefore, be checked by a second estimation in which the number of shakings with petroleum are increased two or three fold. Leathes * has modified and considerably improved this method.

Kumagawa and Suto † have found that the following method gives good results: Two to five grams of the dry substance ‡ are heated on a water bath for two hours with 25 c.c. of 5 N sodium hydroxide (20 grams in 100 c.c.) in a covered beaker. The mixture is then transferred to a separating funnel and acidified with 30 c.c. of 20 per cent hydrochloric acid. The fatty acids set free are taken up with ether, and the ethereal solution is filtered through asbestos and evaporated. The residue, which contains colouring matter, lactic acid and other substances as well as fatty acids, is dried for some hours at 50°, and then taken up with light petroleum, whereupon the impurities separate out in resinous form. After filtering through asbestos the petroleum is distilled off, and the residue, consisting of almost pure fatty acids, is dried at 50° to constant weight.

### QUANTITATIVE METHODS EMPLOYED FOR THE CHARACTERIZATION OF FATS.

The following estimations are in common use for the commercial valuation of fats:—

#### (1) *The Acid Number.*

This is the number of milligrams of potassium hydroxide required for the neutralization of the *free* acids in a sample of fat.

* Leathes: "The Fats," London, 1910.

† Kumagawa and Suto: "Biochem. Zeit.," 1908, **8**, 212.

‡ Yoshitaka Schimidzu ("Bioch. Zeit.," 1910, **28**, 237) recommends using undried material since drying leads to a loss of fat, probably from oxidation.

This number is determined by dissolving 1 or 2 grams of the sample in 15 or 20 c.c. of a mixture of 1 part of alcohol with 2 parts of ether, and titrating the solution with N/10 alcoholic potash in the presence of phenolphthalein.

### (2) *The Saponification Value.*

This is the number of milligrams of potassium hydroxide required for saponifying 1 gram of the fat.

From 1 to 2 grams of the sample are weighed out into a 250 c.c. conical flask; 25 c.c. of approximately seminormal alcoholic potash are then added, and the flask is attached to a reflux condenser and heated over a water bath for about half an hour; the solution is then diluted with 25 c.c. of water and cooled, then the excess of potash is titrated back by means of N/2 hydrochloric acid. In order to determine the strength of the alcoholic potash 25 c.c. of it are heated at the same time under exactly similar conditions in a second conical flask, but without any fat; in this way any error due to the effect of the alkali on the glass vessel is eliminated. The difference in the two titration readings gives the amount of acid equivalent to the potash used up in saponifying the fat, from which the number of milligrams of alkali required for 1 gram of fat may be calculated.

Since one molecule of any monobasic acid requires one molecule of potash, the magnitude of the saponification value is inversely proportional to the molecular weight of the acids contained in the fat.

| | Molecular Weight. | Saponification Value. |
|---|---|---|
| Butyrin | 302 | 557·3 |
| Palmitin | 806 | 208·8 |
| Stearin | 890 | 189·1 |
| Olein | 884 | 190·4 |
| Coco-nut oil | — | 246-260 |
| Palm-kernel oil * | — | 242-250 |
| Palm oil † | — | 196-202 |
| Olive oil | — | 185-196 |

### (3) *Iodine Value.*

It was first observed by Hübl that an alcoholic solution of iodine containing mercuric chloride reacted at ordinary temperatures both with the free unsaturated acids and with

* The oil contained in the kernel of the palm fruit.
† The oil contained in the fleshy part of the fruit.

their glycerol esters the fats. By elaborating the reaction, Hübl converted it into one of the most valuable criteria at present known for the detection and estimation of unsaturated acids in fats, and the so-called "iodine value" provides an excellent method of characterizing a fat.

For the determination of the iodine value of a fat the following solutions are required:—

(*a*) An *iodine solution* made by mixing together equal volumes of two substances containing respectively 25 grams of iodine in water and 30 grams of mercuric chloride in 500 c.c. of 96 per cent alcohol. The two solutions should be mixed together about twenty-four hours before use, as the resulting mixture alters its strength considerably during the first few hours after it has been made.

(*b*) A sodium thiosulphate solution containing roughly 24 grams of crystallized salt in 1 litre of water; the strength of this solution is accurately determined as follows: Twenty c.c. of a potassium bichromate solution containing 3·8657 grams of the pure salt dissolved in 1 litre of water are run into a stoppered bottle containing 10 c.c. of a 10 per cent solution of potassium iodide and 5 c.c. of concentrated hydrochloric acid. The resulting brown solution, if carefully made, should contain exactly 0·2 gram of iodine; it is at once titrated by means of the thiosulphate solution, and, supposing $x$ c.c. were required to decolorize it then it follows that 1 c.c. of thiosulphate is equivalent to $\frac{0{\cdot}2}{x}$ gram of iodine.

(*c*) Chloroform or carbon tetrachloride, the purity of which should be tested by mixing 20 c.c. of it with 20 c.c. of the iodine solution and titrating the free iodine two or three hours after; the amount found should be exactly the same as that contained in 20 c.c. of the iodine solution to which no chloroform or carbon tetrachloride has been added.

(*d*) A 10 per cent solution of potassium iodide made by dissolving 1 part of the iodide in 10 parts of water.

(*e*) A starch solution freshly prepared by boiling up a suspension of 0·5 gram of starch in 50 c.c. of water.

The determination of the iodine value is carried out as follows:—

From 0·15 to 0·18 gram of a drying or marine animal oil,

0·2 to 0·3 gram of a semi-drying oil, 0·3 to 0·4 gram of a non-drying oil or 0·8 to 1·0 gram of a solid fat are accurately weighed from a weighing bottle by difference into a 500-800 c.c. bottle, provided with a well-ground stopper, and dissolved in 10 c.c. of the chloroform (*c*); 25 c.c. of the iodine solution (*a*) are then run in, and the stopper, which is moistened with potassium iodide solution (*d*) to prevent loss of iodine by volatilization, is inserted. If a clear solution is not obtained more chloroform must be added. The bottle is then left to stand in the dark and if the dark brown colour should disappear after two hours or less, another 25 c.c. of the iodine solution must be added, as it is essential that there should be a considerable excess of iodine. In the case of solid fats and non-drying oils the reaction can be considered as being complete after six to eight hours, but in the case of drying oils or fish oils twelve to eighteen hours are necessary. After the completion of this time from 15 to 20 c.c. of the potassium iodide solution (*d*) are added, and, after thorough shaking, the mixture is diluted with 400 c.c. of water. If a red precipitate of mercuric iodide is produced, more potassium iodide solution should be added. The excess of free iodine, part of which is dissolved in the chloroform and part in the potassium iodide solution, is then titrated by shaking with the standardized sodium thiosulphate solution until only a faint yellow colour remains. A little of the starch solution is now added and the titration is continued until the dark blue colour is destroyed. Twenty-five c.c. of the original iodine solution are then titrated in a similar way with the sodium thiosulphate, and the difference in the two results gives the amount of iodine absorbed. The amount of iodine thus absorbed by 100 grams of the fat gives the *iodine value.*

The values obtained by the Hübl method are generally considered to be very reliable and concordant, but the method is somewhat tedious, and for this reason the more rapid method of Wijs * is preferable.

The iodine solution required for this method is obtained by separately dissolving 9·4 grams of iodine chloride and 7·2 grams of finely powdered iodine in separate flasks in about 200

* Wijs: "Zeit. anal. Chem.," 1898, 277; "Zeit. Unters. Nahr. Genussm.," 1902, 497.

c.c. of gently warmed glacial acetic acid. The two solutions are then united in a 1 litre graduated flask and made up to the mark with more glacial acetic acid.

This solution should be standardized on the following day by mixing 20 c.c. of it with 10 c.c. of 10 per cent potassium iodide solution and titrating the free iodine by means of the standard thiosulphate.

The actual determination of the iodine value is performed as follows:—

From 0·2-0·4 gram of fat should be carefully weighed and dissolved in 10 c.c. of pure carbon tetrachloride (which has been shown by a blank test not to absorb iodine); 25 c.c. of the iodine solution are then added and the flask is stoppered and set aside in the dark for one or two hours. The liquid is then transferred to a larger flask, the smaller flask being washed out thoroughly by means of 10 c.c. of potassium iodide solution and water until the total volume is about 300 c.c. The solution is then titrated with the thiosulphate. The difference between this reading and the amount required by 25 c.c. of the iodine solution is a measure of the iodine absorbed by the amount of fat.

The values obtained by Wijs's method are, as a rule, rather higher than those obtained by the Hübl method.

Appended is a list of iodine values of some important fats.

| | |
|---|---|
| (*a*) Drying Oils— | |
| Linseed oil | 173-201 |
| Hemp-seed oil | 148 |
| Sunflower oil | 119-135 |
| Pine-seed oil | 101-103 |
| (*b*) Semi-Drying Oils— | |
| Beech-nut oil | 104-111 |
| Cotton-seed oil | 108-110 |
| Sesame | 103-108 |
| Rape oil (colza) | 94-102 |
| (*c*) Non-Drying Oils— | |
| Almond oil | 93-97 |
| Olive oil | 79-88 |
| Grape-seed oil | 96-142 |
| Castor oil | 83-90 |
| (*d*) Vegetable Fats— | |
| Cacao butter | 32-41 |
| Palm-kernel oil * | 13-17 |
| Coco-nut oil * | 8-10 |

* Though described as oils these substances are both solid at ordinary temperatures, melting at about 25°.

### (4) *The Reichert Meissl Value.*

This represents the number of cubic centimetres of N/10 caustic potash required for neutralizing the volatile acids liberated from 5 grams of a sample of fat under certain special conditions.

The determination is carried out as follows: Five grams of the sample are weighed into a 200 c.c. flask and saponified by warming with 70 c.c. of 10 per cent alcohol and 2 grams of caustic potash. The excess of alcohol is then evaporated off and the residue, after dissolving in 100 c.c. of water, is acidified with 40 c.c. of sulphuric acid (1 : 10); a few chips of asbestos are then dropped into the flask and the liquid is distilled through a Liebig condenser at such a rate that exactly 110 c.c. of distillate pass over in an hour. 100 c.c. of the distillate remaining after filtration are titrated with N/10 caustic potash in the presence of phenolphthalein. Appended are the numbers obtained for several different fats:—

| | | | |
|---|---|---|---|
| Palm-oil | 5·6·8 | Lard | 0·68 |
| Coco-nut oil | 6·6-7·0 | Tallow | 0·5 |
| Linseed oil | 0·0 | Goose fat | 0·2-0·3 |
| Olive oil | 0·6 | Butter fat | 20·6-33·1 |

The determination of the Reichert Meissl value is of considerable value for the detection of adulteration in butter, since any adulterant will at once lower the value.

### (5) *The Acetyl Value.*

This is a measure of the amount of hydroxyl groups which a fat contains; its value depends upon the fact that compounds containing an alcoholic hydroxyl group react with acetyl chloride or acetic anhydride so as to replace the hydrogen of the hydroxyl by the acetyl group ($CH_3CO-$) as shown by the equation:—

$$ROH + \begin{matrix} CH_3CO \\ CH_3CO \end{matrix}\!\!>O = ROCOCH_3 + CH_3COOH$$

If the resulting acetyl derivative is saponified by means of caustic potash it breaks up as follows:—

$$ROCOCH_3 + KOH = ROH + CH_3COOK$$

and it is possible to determine the number of milligrams of

caustic potash which are thus utilized in combining with the acetyl groups to form potassium acetate.

The number of milligrams of potash required by the acetyl derivative obtained from 1 gram of the fat is termed the acetyl value of that fat.

Castor oil and grape-stone oil have particularly high acetyl values owing to the large proportion of hydroxyacids which they contain.

The following are the acetyl values of some of the more important oils, fats and waxes :—

| | | | |
|---|---|---|---|
| Linseed oil . | 3·98 | Castor oil . | 153-156 |
| Olive oil . | 10·64 | Grape-seed oil | 144 |
| Rape-seed oil | 14·7 | Carnauba wax | 55·24 |
| Palm oil . | 18·0 | Lard . . | 2·6 |
| Palm-nut oil . | 1·9-8·4 | Butter . . | 1·9-8·6 |

The following method, due to Lewkowitsch, has been adopted as the standard process.

About 10 grams of the fat are boiled in a round-bottomed flask under a reflux condenser for two hours with twice their weight of acetic anhydride. The mixture is then poured into a litre flask and boiled for half an hour with 500-600 c.c. of water, a slow stream of carbon dioxide being conducted into the liquid all the while to prevent bumping. After cooling, the upper layer of water is siphoned off and the lower oily layer is again boiled with water as above, the whole process being repeated three times. The oil is finally filtered and washed on the filter paper with boiling water until the filtrate is no longer acid, whereupon it is dried in an oven and weighed.

About 5 grams of the acetylated product are next saponified by boiling with alcoholic potash * as described under the determination of the saponification value. The alcohol is then evaporated off, and the resulting soap is dissolved in water.

Dilute sulphuric acid (1 : 10) is then added in excess and the solution is steam distilled until 600-700 c.c. of water have passed over. The distillate is titrated with N/10 caustic potash using phenolphthalein as indicator; the number of cubic centi-

* Prepared by dissolving about 32 grams of 90 per cent stick potash in the least quantity of water and diluting to 1 litre with 96 per cent alcohol; the solution should be filtered after standing for twenty-four hours.

metres required for neutralization multiplied by 5·61 and divided by the weight of fat taken gives the acetyl value.

### PHYSIOLOGICAL SIGNIFICANCE OF FATS.

The great function of fats in the economy of the plant is connected with nutrition. They form one of the most important food reserves of plants, and as such may occur in vegetative or in propagative organs.

With regard to their origin in plants very little is known; they first appear as very small vacuoles in the protoplasm which eventually run together forming large drops.

In some cases oil has been described as owing its origin to the activity of elaïoplasts, which are colourless bodies of various shapes usually grouped around the nucleus, and, like other plastids, of a protoplasmic nature. They are, or have been, supposed to act with regard to oil formation much as leucoplasts do with respect to starch formation. Elaïoplasts have been observed in many Monocotyledons such as *Vanilla*, *Funkia*, *Gagea*, *Ornithogalum*, etc., in the flower of a Dicotyledon, *Gaillardia Lorenziana*, and in *Psilotum*.

The development of the elaïoplasts of *Gaillardia* has been followed by Beer,* who found that they are formed by the aggregation of chloroplasts which then degenerate and give origin to the oil. He considers it is most unlikely that elaïoplasts perform any function of direct importance to the life of the plant, although they may in some cases, the corolla-hairs of *Gaillardia*, for instance, serve a biological purpose.

But although elaïoplasts may not perform the function originally ascribed to them, it does not necessarily follow that fats, more especially when occurring in the green parts of plants, may not be direct photosynthetic products. Thus Fleissig considers that in the case of *Vaucheria*, a plant which contains an abundance of fat, this substance is a direct photosynthetic product comparable to the starch and sugar in ordinary green leaves. On the other hand, it is, of course, possible that the fats in such cases may have been produced by secondary changes in the original product of photosynthesis.

In many cases there can be but little doubt that fats are

* Beer: "Ann. Bot.," 1909, **23**, 63.

produced from carbohydrates; the work of Schmidt,* Le Clerc du Sablon,† and others has shown that as the carbohydrates disappear so fats appear. For example, in the case of the almond the seeds when they begin to ripen are rich in carbohydrates and poor in fats, whereas the reverse is true when they are fully matured. The same holds true for the seeds of *Ricinus* and *Pæonia*. The nature of the carbohydrates used up in this process varies in different plants; thus it is stated that in the case of the olive mannite is the carbohydrate. This statement, due to de Luca, is not accepted by other investigators of the same plant; according to Funaro the mannite does not appear until after the oil has been formed.

In the case of *Ricinus* seeds the oil is formed from glucose, and in *Pæonia* principally from starch. The facts that fat may be translocated as such, provided it be an emulsion sufficiently fine, or in the form of fatty acid or glycerine, suggest that the fats in seeds have not been formed *in situ*, but have been conveyed there. This may be true to a certain extent, but consideration of the fact that fat will appear as the carbohydrates disappear in immature seeds removed from the parent plant, together with the facts relating to the formation of fats in vegetative organs under the influence of cold (p. 2), leads to the conclusion that the substances in question are formed at the expense of carbohydrates. Further, corroborative evidence is afforded by well-ascertained facts relating to similar problems in animals.

Ivanow,‡ experimenting with rape seed, has shown that they contain a lipase which may either hydrolyse a fat or may synthesize one from fatty acid and glycerine. Thus, if a glycerine extract of the seed be mixed with oleic acid, fat is synthesized, but, on diluting with water, the fat is split up again. This same author § has published important observations on the synthesis of fats in oily seeds mainly from the carbohydrates glucose, sucrose, and starch. These substances are synthesized in the order given, the last two being first

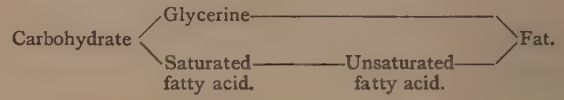
* Schmidt: "Flora," 1891, **74**, 300.

† Le Clerc du Sablon: "Compt. rend.," 1893, **117**, 524; 1894, **119**, 610; 1896, **123**, 1048; "Rev. Gen. Bot.," 1895, **7**, 145; 1897, **9**, 313.

‡ Ivanow: "Ber. deut. bot. Gesells.," 1911, **29**, 595.

§ *Ibid.*, "Beith. bot. Centr.," 1912, **28**, 159.

hydrolysed. The initial acids to be formed are characterized by a low iodine value, showing that they are saturated. Further, since the Reichert Meissl value is constant and does not vary with the acid number, it is concluded that the acids first formed belong to the higher members of the fatty series. The saturated acids are followed by the unsaturated. Ivanow gives the following scheme to indicate the essential stages in the synthesis of fat in a typical instance such as the seed of flax :—




During the germination of oily seeds a reversal of this process takes place. The work of Schmidt, Green,* Le Clerc du Sablon, and others, has shown that the first process is that of hydrolysis which splits the fat into a fatty acid and glycerine, lipase being the active agent.

Thus in the sunflower Miller† found that less than 1 per cent of free fatty acid was present in the oil of the cotyledons of the resting seed; as germination proceeded there was a gradual increase, thus the ether extract of the cotyledons of a seedling in which the plumule was just showing contained 30 per cent of fatty acid.

The presence of the acid may be demonstrated in such germinating seeds, but the same statement does not hold for glycerine, probably because it is translocated with great rapidity, and is quickly transformed. There can, however, be no doubt that this substance is formed because if, for example, castor oil be subjected *in vitro* to the action of lipase obtained from *Ricinus* seeds, the presence of glycerine may be detected with ease.

With regard to other changes which the original fat undergoes during germination, Schmidt found that the iodine number of the unsaturated acids and oils decreased during germination, which indicates that saturation of the acid radicles takes place. This is controverted by von Fürth,‡ who found

* Green: "Proc. Roy. Soc., Lond.," 1890, **48**, 370.
† Miller: "Ann. Bot.," 1910, **24**, 693.
‡ Von Fürth: "Hofm. Beitr. Chem. Phys. Path.," 1904, **4**.

no change in the iodine value. The observations of Schmidt, however, have been corroborated by Miller, who found that in *Helianthus annuus* the iodine value decreased from 136·2 for the seed to 67·4 for a seedling with the plumule just elongating.

Further corroboration is given by Ivanow* who, for his study on the transformation of fats during germination, selected flax, hemp, rape, and poppy seeds, since each is characterized by the possession of fats rich in acids of a specific series. Thus the oil of hemp seed is rich in acids of the unsaturated linolenic series, whilst poppy-seed oil is rich in acids of the saturated fatty acid series.

By ascertaining the iodine and other values of the fats of these seeds at different periods of germination, it was found that the acids disappeared in the sequence linolenic, linolic, oleic, and, finally, palmitic; in other words, the acids were consumed at a rate inversely proportional to their degree of saturation.

Ivanow considers that the fall in the iodine value of the fats is due rather to the rapidity with which the more unsaturated fatty acids are used up in the formation of carbohydrates rather than to their oxidation. He further found that the saturated fatty acids not uncommonly exist in a free state whilst the unsaturated acids occur in the form of glycerides.

Von Fürth also found that during germination the acetyl value decreased from 87·5 in the resting seed to 50·5 in the young seedling, from which he concluded that the normal fatty acid does not change into hydroxy fatty acid. Also, he could find no proof of the fatty acid breaking down into simpler substances as indicated by the molecular weight remaining practically constant.

This hydrolysis is the first action, but it is not the final one since carbohydrates quickly appear during the germination of such seeds. Since the days of de Saussure, who was the first to draw attention to this phenomenon, much evidence relative to this carbohydrate formation has accumulated.

In the case of *Ricinus* le Clerc du Sablon found that the resting seed contained 69 per cent of oil and 4 per cent of sugar, but in a seedling 11 cm. high the oil had fallen to 11 per

* Ivanow: "Jahrb. wiss. Bot.," 1912, 50, 375.

cent and the sugar had risen to 14 per cent. It was further found that the sugar contained in the resting seed has a slight excess of non-reducing sugar, which increased more rapidly than the reducing sugar; finally, however, the latter variety preponderated.

Le Clerc du Sablon also found the same relation between oil and sugar to obtain during germination of rape, hemp, poppy, almond, and walnut.

Similar observations have been made by Green and Jackson,* who found that in the resting seed of *Ricinus* the most abundant sugar is sucrose, which gives place to invert sugar in the early stages of germination. Subsequently the sucrose increases in amount, and occurs in quantities greater than the invert sugar; thus there is reason for supposing that the sucrose is a temporary reserve food.

The following table which summarizes the changes in the sugar content is taken from Green and Jackson's paper :—

| Time of germination in hours. | Invert sugar in milligrams. | Cane sugar in milligrams. |
|---|---|---|
| 0 | 1·1 | 10·7 |
| 45 | 2·7 | 5·17 |
| 69 | 2·3 | 0 |
| 117 | 6·7 | 19·4 |
| 168 | 5·2 | 10·5 |
| 216 | 19·5 | 35·7 |
| 240 | 29·01 | 35·8 |
| 312 | 40·8 | 52·6 |

Miller has found that in the sunflower, *Helianthus annuus*, the amount of ether extract of the cotyledons diminishes gradually from the beginning of germination, the most rapid depletion occurring during the period between the first appearance of the seed-leaves above ground and the point of full expansion. Also, the greatest increase in the hypocotyl and roots coincides with the period of maximum depletion from the seed-leaves. With regard to the sugar content, Miller states that the resting embryo contains about 4 per cent of sucrose, during germination there is a decrease, and this is followed by a gradual increase until the seed-leaves begin to unfold. Up to this stage the cotyledons contain only a non-reducing sugar, but as

* Green and Jackson: "Proc. Roy. Soc., Lond.," B., 1906, 77, 69.

the seed-leaves assume the functions of foliage leaves a reducing sugar appears, and, in a short time, is the only sugar present. In the hypocotyl and roots the amount of sugar rapidly increases until in seedlings about 4 inches long it may amount to 20 per cent of the dry weight, then a gradual decrease takes place. There is also a small increase in the amount of starch.

The nature of the carbohydrate differs in different plants; thus in addition to the above-mentioned plants, during the germination of *Allium* and of *Cucumis* much glucose makes its appearance; this is also true, although to a lesser degree, for *Cannabis sativa*, in which case the glucose is quickly transformed into starch.

In other instances starch is said to be the carbohydrate formed.

It is thus seen that there is an intimate connexion between carbohydrates and oil, and the question naturally arises how is the one connected into the other, to which there is no answer.

The consideration of the formulæ of the substances in question shows that fats poor in oxygen give rise to carbohydrates rich in oxygen, and vice versa; but as to how this is accomplished nothing of a definite nature is known.

Many suggestions have been put forward, and before mentioning these the reader may be reminded of the large amount of oxygen which is absorbed during the germination of oil-containing seeds.

Detmer considered that starch may arise from the free oleic acid according to the equation:—

$$C_{18}H_{34}O_2 + 27O = 2\,(C_6H_{10}O_5) + 6CO_2 + 7H_2O$$

According to Maquenne the sugar has an origin depending upon the nature of the oil; thus if the fat be saturated, of its hydrolytic products the glycerine gives rise to the sugar, whilst the fatty acids are used up in oxidative processes. If, on the other hand, the fat be unsaturated, the fatty acid contributes to the formation of the sugar.

This change is supposed to be effected by the oxidation of the chain at the double bond setting free two unsaturated groups which by polymerization give rise to sugar.

These conclusions are based on the observations that during the germination of the seeds of *Arachis* the carbohydrate increases to 5·6 per cent of the dry weight, whilst in *Ricinus* the increase is 10 per cent. The glycerine of the fat would be sufficient to form about 5 per cent of carbohydrate; this roughly was the amount observed in the case of *Arachis*, but in *Ricinus* the amount was about three times as great.

Mazé has put forward the suggestion that the transformation of oil into sugar is effected by an enzyme.

It has already been mentioned that glycerine so far has not been demonstrated in germinating fatty seeds; this may be owing to its powers of rapid diffusion or to the fact that it is used up in the synthesis of other substances. Thus it has just been mentioned that Maquenne thought that it might be the origin of the sugar in some cases at any rate; Green originally thought that such was the case in *Ricinus*, an opinion which he no longer holds. Green and Jackson state that there is reason to suppose that the protoplasm of the endosperm of *Ricinus* is increased at the expense of the initial reserve food-materials; subsequently, further carbohydrates for the nutrition of the embryo are formed by the activity of this protoplasm: in other words, these authors do not consider that the increase in sugar during germination and the decrease in oil are directly associated; the disappearance of the latter and the formation of the former are "features of a new metabolism set up in the cells as germination becomes established". Also they express the opinion that the glycerine may be used up in the formation of lecithin.

Le Clerc du Sablon has put forward the idea that there might be present an enzyme which acts on the fat without liberating the glycerine.

These views are concerned chiefly with the formation of carbohydrates from fats; a reversal of the process might or might not explain the formation of fats from carbohydrates.

The whole question is of considerable difficulty and, of course, refuge may be taken in the hypothesis first put forward by Nägeli that the fats are products of the disintegration of the protoplasm. Thus the carbohydrates might be assimilated by the protoplasm which might produce the oil by some catabolic process.

With regard to the possible formation of fats from proteins very little information is available. On the animal side there is some evidence to show that substances derived from proteins may be so utilized; a possible connexion may be found in the phospholipines (phosphatides) which are compounds of fatty acids containing either nitrogen or phosphorus, or both.

Leathes* points out that the fatty acid may be formed from glucose by processes analogous to the synthesis of butyric acid from lactic acid which in turn is formed from the glucose. For the underlying reasons, which are rather too complicated to be dealt with here, Leathes's monograph must be consulted. It may, however, be pointed out in this connexion that the investigations of Hanriot are very significant; he found that, in attempting the oxidation of fat *in vitro*, 15 per cent of its weight of oxygen was absorbed, and in the products of its oxidation butyric and acetic acids occurred, but no carbohydrate.

In conclusion brief mention may be made of Schmidt's views regarding the translocation of fats. He considers that in many cases the oils may be transported as such to those organs requiring it, for he found that the amount of fatty acid present in the germinating seeds was smaller than would be supposed if it were hydrolysed before translocation, also that neutral oil appears in regions of the plant removed from the storage organ.

He considers the walls of cells are permeable to oil; provided it be an emulsion sufficiently fine, and especially if a free fatty acid be present, the permeability being directly proportional to the amount of such acid present. It is thought that the acid forms a soap in the walls, and thus facilitates the passage.

It is not improbable that both methods are adopted by the plant, viz. the translocation of the products of the dissociation of the fat, and the translocation of oil *qua* oil.

* Leathes: "The Fats," London, 1910.

## WAXES.

The chief function of waxes in plants is to form a protective covering against undue evaporation of water. They are found most commonly in or on the cuticle of leaves and fruits where they give rise to the glaucous effect.

As already stated, the waxes resemble the fats in their chemical constitution in so far as they are esters, but they differ in the nature of their alcohol constituent which is not glycerol but is usually a monohydric alcohol such as cetyl alcohol $C_{16}H_{33}OH$, carnaubyl alcohol $C_{24}H_{49}OH$, pisangceryl alcohol $C_{24}H_{49}OH$, ceryl alcohol $C_{26}H_{53}OH$, myricyl alcohol $C_{30}H_{61}OH$, cholesterol or phytosterol $C_{27}H_{45}OH$.

In addition to the acids already mentioned as occurring in fats, the following are also met with in waxes in the form of esters: ficocerylic acid $C_{13}H_{26}O_2$, carnaubic acid $C_{24}H_{48}O_2$, and pisangcerylic acid $C_{24}H_{48}O_2$, as well as acids belonging to series of the general formula $C_nH_{2n-2}O_2$ and $C_nH_{2n}O_3$.

The term wax used in the chemical sense has reference only to the chemical composition of these substances, regardless of their physical state of aggregation, and consequently both liquid and solid waxes are known.

Waxes of the former class are, however, only known in the animal kingdom, they are ordinary sperm oil and arctic sperm oil.

Among the better-known vegetable waxes may be mentioned:—

(*a*) *Carnauba Wax* obtained from *Copernicia cerifera;* this wax contains ceryl and myricyl alcohols, and two acids, cerotic acid $C_{26}H_{52}O_2$, and carnaubic acid $C_{24}H_{48}O_2$, together with a hydroxy-acid of the formula $C_{21}H_{42}O_3$.

(*b*) *Pisang Wax* obtained from the leaves of *Cera musae* is the pisangceryl ester of pisangcerylic acid.

The following are some of the more important waxes of animal origin:—

Wool wax, better known as wool fat or lanolin (which is rich in cholesterol), beeswax, spermaceti, and Chinese insect wax.

### PHYSICAL AND CHEMICAL PROPERTIES OF WAXES.

Waxes are soluble in all the ordinary fat solvents such as benzene, ether, chloroform, etc., though they are rather less soluble than the fats.

Being free from glycerides the waxes, when heated, *give no smell of acrolein;* they do not become rancid like the fats, and are less easily hydrolysed, but they can be decomposed by prolonged heating with alcoholic potash.

### FURTHER REFERENCE.

Lewkowitsch: "Chemical Technology and Analysis of Oils, Fats, and Waxes," London, 1915.

## LIPOIDS.

Closely related to the fats is the group of substances known as phosphatides, phospholipins or lecithins, the last name being derived from the Greek λεκιθος, meaning egg yolk, from which substance the first representative of the class was prepared.

The name phosphatide was first given by Thudichum to a number of substances containing phosphorus which he obtained from brain. Subsequently Overton introduced the term lipoid to represent a group of substances occurring in the animal tissues which resembled fats in their solubilities. Leathes uses the term phospholipins, in place of phosphatides, to denote compounds of fatty acids containing phosphorus and nitrogen, and proposes the name of lipins for compounds of fatty acids that contain nitrogen but no phosphorus.*

### OCCURRENCE.

Lecithin compounds occur in the grains of cereals, in the seeds of several Leguminosæ, *Ricinus*, and species of *Pinus;* in the leaves of *Castanea*, and in Fungi; they are also widely distributed in animals. In fact, these substances are stated to occur in small quantities in all living cells, and they appear to be more especially abundant where fats occur. Zlataroff considers that light is requisite for the formation of lecithins since he finds that the amount present in seeds increases during germination in the light.†

* The nomenclature of this group of substances is considered by Maclean: "Lecithin and Allied Substances. The Lipins," London, 1918.

† Zlataroff: "Biochem. Zeit.," 1916, 75, 200.

The approximate amount of lecithin contained in various substances may be seen from the following table:—

| | |
|---|---|
| Egg yolk | 9·4 per cent |
| Liver | 2·1 ,, |
| Blood | 1·8 ,, |
| Leguminous seeds | 0·8-1·64 per cent |
| Cereals | 0·25-0·53 ,, |

Botanically, the term lecithin is generic, and plant products so called have not yet been obtained in a pure state and contain other substances of a similar nature.

### PREPARATION.

The most convenient source for the preparation of lecithin is egg yolk. This substance is extracted with five times its volume of 96 per cent alcohol; the extract is then cooled to 0°, filtered and precipitated with an alcoholic solution of cadmium chloride; the precipitated double salt is next washed with alcohol and ether; it is then decomposed by boiling with eight times its quantity of 80 per cent alcohol and carefully adding a concentrated solution of ammonium carbonate until all the cadmium is thrown out of solution; the solution is filtered whilst hot and on cooling the filtrate to 10° the lecithin is deposited. It may be purified by dissolving in chloroform and precipitating from solution by the addition of acetone in which lecithin is insoluble.

Pure lecithin has not as yet been obtained from vegetable sources, the substances isolated by Winterstein * and his collaborators from wheat flour and from the seeds of *Avena sativa*, *Lupinus albus*, *L. luteus*, *Vicia sativa*, from the leaves of *Æsculus hippocastanum*, etc., being mixtures which, moreover, contain a carbohydrate complex. For an account of the methods employed in the extraction of these substances the original papers should be consulted. Smolensky † found that wheat germs (i.e. the embryos which are a bye-product of the flour mills) yielded a phosphatide whose composition was much closer to that of ordinary lecithin than was that obtained from the flour.

* Winterstein and Hiestand: "Zeit. physiol. Chem.," 1907, **54**, 288; Winterstein and Smolensky: *id.*, 1908, **58**, 506; Winterstein and Stegmann: *id.*, 1908, **58**, 527. See also Schulze and Likiernik: *id.*, 1891, **15**, 405; Schulze: *id.*, 1895, **20**, 228.

† Smolensky: *id.*, 1908, **58**, 522.

REACTIONS AND CHARACTERISTICS.

The following are some of the more characteristic reactions:—

1. If to an alcoholic solution of lecithin an alcoholic solution of cadmium chloride be added, a white precipitate of the cadmium chloride double salt is formed.

2. If a little lecithin is boiled with caustic soda, trimethylamine is formed, and may be identified by its characteristic smell; the solution contains sodium salts of fatty acids; on acidifying with sulphuric acid the fatty acids are precipitated.

3. Lecithin gives a purple coloration when added to a mixture of strong sulphuric acid and sugar solution.

The lecithins are yellow or yellowish-white wax-like solids with a peculiar odour; they are very hygroscopic, but some of them when carefully dried in a vacuum can be obtained in form of powder. They dissolve readily in the ordinary fat solvents, such as ether, chloroform, carbon tetrachloride, benzene, carbon disulphide, and also in oils and fats; they are also soluble in hot alcohol and ethyl acetate, but are only sparingly soluble in acetone and methyl acetate, so that these two substances may be used for the purification of the crude product. They are precipitated from alcoholic solutions by alcoholic solutions of platinic or cadmium chlorides.

When mixed with a small quantity of water they swell up, forming slimy threads, known as myelin forms; with excess of water they "dissolve," forming colloidal solutions which are not coagulated by boiling, but from which they may be precipitated by the addition of certain salts, such as those of barium and calcium.

As already stated, phosphatides dissolve in the same organic solvents as the fats, and are consequently liable to be extracted from the tissues together with fats; this fact must be borne in mind in estimating the amount of fat in any substance by the method of weighing the residue remaining after the evaporation of an ether extract.

The chemical composition of the phosphatides * differs from that of the fats primarily in containing the two elements nitrogen and phosphorus in addition to carbon, hydrogen,

* See Maclean: "Biochem. Journ.," 1915, 9, 351.

and oxygen. According as they contain one or two atoms of phosphorus in their molecules, they are classed as mono- or di-phosphatides. The lecithins, like the fats, are esters of glycerol with higher fatty acids, such as palmitic, stearic, and oleic acids, but they differ from the fats in being at the same time esters of phosphoric acid, as is shown by the following formula of lecithin from egg yolk :—

$$\begin{array}{l} CH_2OCOC_{17}H_{35} \\ | \\ CHO{\cdot}COC_{17}H_{35} \\ | \\ CH_2{\cdot}O{\cdot}P(OH){\cdot}OCH_2CH_2N(CH_3)_3OH \\ \quad\quad \| \\ \quad\quad O \end{array}$$

Lecithin is readily hydrolysed by boiling with alkalis, notably baryta, and is also broken up by lipase, and, less readily, by mineral acids. The products of its hydrolysis are glycero-phosphoric acid :

$$CH_2OHCHOHCH_2OP = (OH)_2$$
$$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad \| \\ \quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad O$$

choline $HON(CH_3)_3CH_2CH_2OH$ and fatty acids ; a similar hydrolysis takes place in the germinating seed.*

Originally it was considered that the fatty acids of lecithin were either stearic, palmitic, or oleic, but it has been found that the iodine values of the acids obtained from lecithin are much higher than would be given by these acids alone.

### CHOLINE.†

To examine the products of the hydrolysis of lecithin, this substance is heated with a solution of barium hydrate in excess ; a baryta soap is formed, which may be filtered off. The aqueous solution contains barium glycero-phosphate and choline ; the latter may be extracted as follows. ‡

Treat the solution with a stream of carbon dioxide until no

* Schulze : "Z. physiol. Chem.," 1887, 11, 365 ; Schulze and Frankfurt : "Ber. deut. chem. Gesells.," 1893, 26, 2151.

† For a fuller account of the methods of isolating and estimating this substance in plant extracts, etc., see Barger : "The Simpler Natural Bases," "Monographs of Biochemistry," London, 1914.

‡ Leathes : "The Fats," "Monographs of Biochemistry," London, 1910.

more barium carbonate comes down. Filter and evaporate the filtrate to dryness. Treat the residue with absolute alcohol, which will dissolve the choline but not the barium glycerophosphate. The alcoholic solution, if treated with an alcoholic solution of platinic chloride, gives a precipitate of the double platinichloride of choline.

Green and Jackson* give the following method: Allow the finely divided material to stand for some days under absolute alcohol. Pour off the extract, and evaporate to dryness; the residue is again extracted with absolute alcohol, and finally with a mixture of alcohol and ether. These extracts are mixed, and the solvents evaporated off. The choline is contained in the residue. The following tests may be employed for its detection:—

1. Boil a strong aqueous solution; decomposition ensues and trimethylamine is given off, which may be recognized by its fish-like smell.

$$\underset{\text{Choline}}{HON(CH_3)_3CH_2CH_2OH} = OHCH_2CH_2OH + \underset{\text{Trimethylamine}}{N(CH_3)_3}$$

2. Add platinic chloride to the aqueous solution; a double platinum salt is formed, which crystallizes on standing. The crystals are soluble in 15 per cent alcohol. Should the crystals not appear, proceed as follows:—

3. Dissolve choline in alcohol and add an alcoholic solution of platinic chloride. Filter off the yellow precipitate, wash with alcohol and dissolve in as little water as possible. Place the solution in a watch glass, and stand in a desiccator. Hexagonal plates will be deposited.

4. In order to detect very small quantities, Rosenheim recommends the following method.† Prepare the double platinum salt, place a drop or two on a glass slip, and allow to evaporate. Add a drop of a solution containing 2 grams of iodine and 6 grams of potassium iodide in 100 c.c. of water, and examine under the microscope. Dark brown prisms or plates will appear and then disappear as evaporation takes place; they will reappear on adding another drop of iodine solution.

* Green and Jackson: "Proc. Roy. Soc., Lond.," B., 1906, **77**, 69.
† Rosenheim: "J. Physiol.," 1905, **33**, 220.

Choline is both a tertiary amine and an alcohol; the aldehyde and acid corresponding to it are both known.

$HON(CH_3)_3CH_2CH_2OH$ Choline — $HON(CH_3)_3CH_2CH(OH)_2$ Muscarine — $HON(CH_3)_3CH_2COOH$ Betaine

The aldehyde, which goes by the name of muscarine, occurs in *Agaricus muscarius;* it is a powerful poison (see p. 274).

By the bacterial decomposition of choline another very poisonous base, neurine, may be obtained; this substance differs from choline by the elements of water.

$HON(CH_3)_3CH_2CH_2OH$ Choline — $HON(CH_3)_3CH{=}CH_2$ Neurine

The choline complex of lecithin on further decomposition can give rise to nitrogen bases, such as dimethylamine $HN(CH_3)^2$ and trimethylamine $N(CH_3)_3$; these substances, which have a fishy smell, also occur in herring brine and are probably there produced from a similar source. They have similarly been obtained from the leaves of *Chenopodium vulvaria*, from the blossoms of *Crataegus Oxyacantha*, from species of *Pyrus*, and from the seeds of *Fagus* (see p. 275).

Lecithins form compounds with sugar, and it is stated that all lecithins of a vegetable origin are in combination with carbohydrates; galactose, glucose and pectose having been identified. The amount of this combined sugar varies pretty considerably; it may be as high as 16 per cent according to Winterstein and Hiestand.*

### *Formation of Lecithin.*

The following table, due to Green and Jackson,† shows the relation between the lecithin, fatty acid, and oil of the endosperm of *Ricinus*, expressed in per cent of weight of the seeds at different stages in their germination :—

| Degree of development. | Oil in seeds. | Fatty acid in seeds. | Lecithin. |
|---|---|---|---|
| Resting seeds . . . . | 82·8 | 2·2 | ·236 |
| Testa just cracked . . . | 67·5 | 4·6 | ·17 |
| Radicle protruding 1-2 cm. . | 52·5 | 11·9 | ·475 |
| Root system established . . | 23·6 | 16·89 | ·873 |

* Winterstein and Hiestand: *loc. cit.* † Green and Jackson: *loc. cit.*

From this it appears that lecithin is formed during germination; although there is, during the early stages of germination, a diminution in the quantity present. It was found when once the maximum was reached that this amount remained constant until the whole of the endosperm was used up.

The products of the decomposition of lecithin, viz. a fatty acid, glycerophosphoric acid, and choline, have been detected by Green and Jackson in the endosperm of germinating seeds of *Ricinus*, and they suppose that the action is reversible, so that the lecithin is formed by the combination of the products of decomposition of the oil and protein reserves of the seeds. Thus the oil provides the fatty acid and the glycerol, of which the latter combines with phosphorus, obtained from the aleurone grains, to form glycerophosphoric acid. The choline is provided by the decomposition of the proteins by means of a tryptic enzyme. But however this may be, in view of our ignorance of these substances, and the fact that vegetable lecithins apparently have seldom or never been obtained in a state of purity, and the uncertainty relating to some of their cleavage products, it does not appear profitable further to consider here the theories which have been advanced to explain their formation.

### *Physiological Significance.*

Nothing of a very definite nature is known of the physiological significance of the lipoids. Overton points out that under certain conditions lecithin and similar substances have the power of absorbing water, and suggests that the ectoplasm may consist of layers of these substances which thus play an important rôle in absorption and secretion. Green and Jackson also consider that it exercises considerable influence on the transport of materials from cell to cell. This view of the lipoid nature of the plasmatic membrane is greatly supported by the work of Czapek * on the surface tension of the external limiting layer of the protoplasm.

Lipoids may of course represent an intermediate product between the fats and proteins, for it is a well-known fact that

* Czapek: "Ueber eine Methode zur direkten Bestimmung der Oberflächenspannung der Plasmahaut von Pflanzenzellen," Jena, 1912.

fats may develop in cheese, but according to Nierenstein * in such cases the fats are derived not from the proteins, but from other substances, such as cholesterol.

Also the view has been put forward that they are the means of setting up the change in zymogens which leads to the formation of enzymes. More recently Palladin † has suggested that there is a relationship between lipoids and respiration, for the more of these substances extracted from seedlings the more was the respiration depressed. Possibly the lipoids, which contain phosphorus, act in a similar way as the phosphates in alcoholic fermentation.

* Nierenstein: "Proc. Roy. Soc., Lond.," B., 1911, **83**, 301.

† Palladin: "Ber. deut. bot. Gesells.," 1910, **28**, 120; Palladin and Stanewitsch: "Biochem. Zeit.," 1910, **26**, 351.

# SECTION II.

## ALDEHYDES.

In view of the important part played by aldehydes in questions relating to the carbohydrates and other compounds, it appears desirable here to draw attention to the chief properties of these substances.

It is well known that the aldehydes are the first products of the oxidation of primary alcohols :—

$$\underset{\text{Methyl alcohol}}{CH_3OH} + O = \underset{\text{Formaldehyde}}{HCHO} + H_2O$$

$$\underset{\text{Ethyl alcohol}}{CH_3CH_2OH} + O = \underset{\text{Acetic aldehyde}}{CH_3CHO} + H_2O$$

The reconversion of formaldehyde into the alcohol can be effected by means of nascent hydrogen obtained by sodium amalgam and water.

Chemically, the aldehydes are very active, undergoing a number of reactions, some of which are of biological significance, whilst others serve as valuable means of isolation or identification.

1. Aldehydes are readily oxidized to the corresponding acids by even such mild oxidizing agents as ammoniacal silver hydroxide or Fehling's solution, or even atmospheric oxygen, as is shown by the following experiments :—

(*a*) A few drops of caustic potash are added to some silver nitrate solution in a test tube, ammonia is then carefully added, drop by drop, until the brown precipitate has just redissolved. A little dilute acetaldehyde solution is poured in and the mixture is warmed gently; if the solution be sufficiently dilute, a silver mirror will be deposited on the side of the test tube; otherwise a black precipitate will be formed :—

$$CH_3CHO + Ag_2O = CH_3COOH + 2Ag$$

(*b*) A little Fehling's solution is gently warmed with a few drops of dilute aldehyde solution; a change in colour takes place, from blue to green and yellow; finally the solution becomes colourless and a red precipitate of cuprous oxide ($Cu_2O$) comes down.

The readiness with which aldehydes are oxidized to acids accounts for the fact that most samples of aldehydes, unless freshly prepared, contain varying amounts of free acid.

2. Aldehydes are readily reduced by nascent hydrogen to the corresponding primary alcohols, according to the equation

$$\underset{\text{Acetic aldehyde}}{CH_3CHO} + 2H = \underset{\text{Ethyl alcohol}}{CH_3CH_2OH}$$

3. Aldehydes restore the colour to Schiff's Reagent (a solution of magenta decolorised by sulphurous acid).

4. Aldehydes when warmed with caustic potash are converted into resinous substances of unknown composition. This can be readily shown with acetaldehyde; formaldehyde, however, when treated with potash undergoes a different change, being converted into a mixture of methyl alcohol and potassium formate, according to the equation

$$2HCHO + KOH = CH_3OH + \underset{\text{Potassium formate}}{HCOOK}$$

5. Aldehydes react with ammonia to form additive compounds; thus acetic aldehyde undergoes the following reaction:—

$$\underset{\text{Acetic aldehyde}}{CH_3CHO} + NH_3 = \underset{\text{Aldehyde ammonia}}{CH_3CHOHNH_2}$$

Here again formaldehyde behaves differently; if ammonia is added to a formaldehyde solution, it is neutralized quantitatively according to the equation

$$\underset{\text{Formaldehyde}}{6CH_2O} + 4NH_3 = \underset{\text{Hexamethylene tetramine}}{(CH_2)_6N_4} + 6H_2O$$

with the formation of a crystalline solid which is used in medicine under the name of urotropine.

The reaction can be employed for estimating * the amount of formaldehyde in a solution by adding a known excess of

* For another method of estimating formaldehyde by weighing the mercury produced by the reduction of an alkaline solution of mercuric sulphite, see Feder: "Archiv. d. Pharm.," 1907, 245, 25.

standardized ammonia solution, and after some time titrating back the excess of ammonia by means of standard acid, using litmus as indicator.

Thus, for example, if 25 c.c. of the formaldehyde solution, after shaking with 50 c.c. of N/2 ammonia, required for neutralization 20 c.c. N/2 hydrochloric acid, then the amount of ammonia used up by the formaldehyde would be 50 – 20 = 30 c.c.

But 30 c.c. N/2 ammonia contain $\frac{30}{1000} \times \frac{17}{2} = \cdot 255$ gram $NH_3$

and since from the equation $4NH_3$ (68) are equivalent to $6CH_2O$ (180)

$$\therefore \cdot 255 \text{ gram } NH_3 \equiv \cdot 68 \text{ gram } CH_2O$$

∴ 25 c.c. of the solution contained 0·68 gram formaldehyde.

6. With sodium bisulphite aldehydes form crystalline addition compounds which, being sparingly soluble in water, can be used for isolating aldehydes from mixtures.

Thus if some saturated sodium bisulphite solution be added to a fairly strong solution of aldehyde and the mixture shaken vigorously, a rise in temperature takes place accompanied by the formation of a white crystalline precipitate :—

$$CH_3CHO + HNaSO_3 = CH_3CHOHSO_3Na$$

7. Aldehydes also form additive compounds with hydrogen cyanide; these compounds are known as hydroxycyanides or cyanohydrins :—

$$CH_3CHO + HCN = CH_3CHOHCN$$

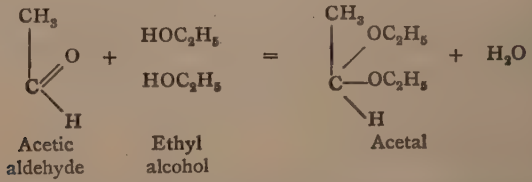
Acetic aldehyde cyanohydrin

8. Aldehydes form crystalline compounds with hydroxylamine, phenylhydrazine, and semicarbazide; in all cases water is split off between the two reacting substances.

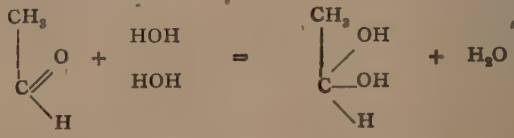
$$CH_3CHO + NH_2OH = CH_3CH : NOH + H_2O$$
$$CH_3CHO + C_6H_5NHNH_2 = CH_3CH : N \cdot NHC_6H_5 + H_2O$$

The resulting compounds, which are known as oximes, hydrazones or semi-carbazones, are usually substances with a characteristic crystalline form and melting point, which may be employed for the identification of the corresponding aldehydes. The use of phenylhydrazine for the identification of the sugars has already been described.

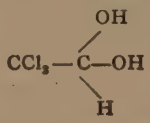
9. The aldehydes are able to react with alcohols with the formation of condensation compounds known as acetals; thus, for example, acetic aldehyde reacts with ethyl alcohol as follows:—




By analogy, acetic aldehyde should also be able to react with water as follows:—




This substance does not, however, actually exist, since a compound having two or more hydroxyl groups attached to the same carbon atom is, as a rule, unstable, and at once loses water. Exceptions to this rule are, however, occasionally met with; for example, chloral $CCl_3CHO$ forms a stable compound, chloral hydrate, of the formula



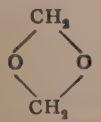

10. Aldehydes exhibit a tendency to polymerize, that is, for two or more molecules to combine together to form new compounds of higher molecular weight.

Thus two molecules of formaldehyde will combine together, forming a compound known as paraformaldehyde $(CH_2O)_2$; this substance, which is a white solid, is obtained by evaporating an aqueous solution of formaldehyde.

A second polymer formed from three molecules of formaldehyde is known as metaformaldehyde or trioxymethylene $(CH_2O)_3$. This substance is produced by the spontaneous polymerization of anhydrous formaldehyde.

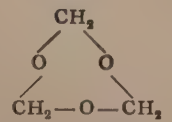
In the case of both the above polymers the molecules of

formaldehyde are probably connected together through oxygen atoms as under :—




Paraformaldehyde

and




Trioxymethylene or
Metaformaldehyde

which accounts for the fact that they are readily broken up into the simple molecules of formaldehyde by heating.

11. A different type of polymerization, involving the linking together of molecules of formaldehyde through carbon, is also known; this type of polymerization, which is sometimes known as aldol condensation, results in the formation of a more stable complex which cannot be reconverted into the simple substance.

The reaction takes its name from the substance produced by the action of dilute hydrochloric acid or zinc chloride on acetic aldehyde.

$$CH_3CHO + CH_2CHO = CH_3CHOH.CH_2.CHO$$
Aldol

The analogous reaction with formaldehyde is, however, brought about by dilute alkalis; in this way two molecules of formaldehyde give rise to glycollic aldehyde,

$$HCHO + HCHO = CH_2OH.CHO$$
Glycollic aldehyde

or three molecules may combine together to produce glyceric aldehyde,

$$HCHO + HCHO + HCHO = CH_2OH.CHOH.CHO$$
Glyceric aldehyde

By repeatedly shaking a 4 per cent solution of formaldehyde for half an hour with an excess of lime water, and then filtering the solution and setting it aside for some days until the odour of formaldehyde had disappeared, Loew * was able to obtain a crude mixture of sugars called formose, from which true reducing hexose sugars have been isolated. This change may be represented by the equation :—

$$6HCHO = C_6H_{12}O_6$$

* Loew : "Ber. deut. chem. Gesells.," 1887, **20**, 142, 3039; 1888, **21**, 270; 1889, **22**, 470, 878.

Similarly H. and A. Euler* have shown that when a 2 per cent solution of formaldehyde is heated for some hours with calcium carbonate, a pentose sugar—arabinoketose—is produced; in addition to this substance, glycollic aldehyde and dihydroxyacetone are produced, but in smaller quantity.

### FORMALDEHYDE.

From the point of view of photosynthesis formaldehyde is of outstanding interest; as is well known, it is at ordinary temperatures a colourless gas with a pungent odour; when cooled to $-21^{\circ}$ it condenses to a liquid. It is usually met with in the form of an aqueous solution, commercial formalin, which contains about 40 per cent of the gas dissolved in water and is used as a disinfectant or as a hardening medium for pathological and other specimens and occasionally as a preservative for milk. It undergoes most of the general reactions for aldehydes which have been mentioned above.

Its peculiar behaviour towards ammonia, resulting in the formation of hexamethylene tetramine, has already been mentioned; this substance, which is used under the name of urotropine, is a crystalline base which dissolves in hot or cold water; with bromine it forms an additive compound—tetrabromo-hexamethylene tetramine $(CH_2)_6N_4Br_4$—which has been used for detecting small quantities of formaldehyde in solution.

Formaldehyde also reacts with ammonium salts as well as with free ammonia, as follows:—

$$6CH_2O + 4NH_4Cl = \underset{\text{Hexamethylene tetramine}}{(CH_2)_6N_4} + 6H_2O + 4HCl$$

This reaction has been made use of as a means of estimating ammonium salts in solution by titrating the amount of free acid liberated according to the above equation on adding sufficient formaldehyde to a solution containing ammonium salts. For this purpose both the formaldehyde solution and the solution to be analysed must be previously neutralized, if necessary. An excess of the neutralized formaldehyde solution is then added to a known volume of the solution containing the ammonium salts, and after thoroughly shaking for one or two

* Euler, H. and A.: "Ber. deut. chem. Gesells.," 1906, 39, 36, 39.

minutes the amount of acid set free is determined by titration with standard caustic soda, using methyl orange as indicator; the amount of ammonia can be calculated from the fact that each 36·5 grams of hydrochloric acid liberated correspond to 17 grams of ammonia.

The reactions most suitable *for characterizing small quantities of formaldehyde* are as follows:—

Rimini's test consists in adding 2 drops of phenylhydrazine hydrochloride, 2 drops of sodium nitroprusside solution, and 1 c.c. of sodium hydroxide to 1 c.c. of the liquid to be tested. A blue colour is formed, which changes rapidly through green and brown to red. Schryver* has modified this test and made it much more sensitive; he recommends the following method: to 10 c.c. of the liquid to be tested add 2 c.c. of a 1 per cent solution of phenylhydrazine hydrochloride freshly made up and filtered; then add 1 c.c. of a 5 per cent solution of sodium ferricyanide, also freshly made up, and 5 c.c. of hydrochloric acid; a brilliant magenta colour is produced. The test is a very delicate one and will detect quantities of formaldehyde varying from 1 part in 1,000,000 to 1 part in 100,000. Acetic aldehyde gives no colour with this reagent.

The following test, due to Denigés,† is sensitive for formaldehyde, even in presence of acetic aldehyde up to 2 per cent; 5 c.c. of an aqueous solution of formaldehyde are mixed with 1·2 c.c. of pure sulphuric acid (sp. gr. 1·66) and 5 c.c. of Schiff's reagent. An intense violet colour having an absorption band in the orange is produced. Schiff's reagent may be prepared by adding a litre of 0·01 per cent of solution of magenta to 20 c.c. of sodium hydrogen sulphite solution (sp. gr. 1·3), and after five minutes adding 20 c.c. of hydrochloric acid (sp. gr. 1·18).

Kimpflin‡ tested for formaldehyde in the leaf of *Agave mexicana* by injecting into it, by means of a capillary tube, a concentrated solution of sodium hydrogen sulphite, containing an excess of *p*methylamino-*m*cresol. The presence of formaldehyde was indicated by the formation of a red precipitate on exposure to light. The precipitate is best seen

* Schryver: "Proc. Roy. Soc. Lond.," B., 1910, **82**, 226.
† Denigés: "Compt. rend.," 1910, **150**, 529
‡ Kimpflin: *id.*, 1907, **144**, 148.

by examining a section of the leaf which has been dipped in absolute alcohol. Formaldehyde is the only aldehyde giving a stable red colour with the above reagent, but other aldehydes give unstable green, yellow, or reddish-brown colours.

*Occurrence in the Plant.*—Since the work of Reinke, many have reported the occurrence of formaldehyde in the plant,* and its presence has been accepted as evidence of the truth of Baeyer's hypothesis of photosynthesis. Recent investigations, however, show that this formaldehyde is a degradation product of chlorophyll. Ewart's † conclusion that chlorophyll contains formaldehyde in a combined state has been confirmed by Schryver,‡ who has published certain observations on the subject. He finds that formaldehyde is more abundant in chlorophyll films after exposure to bright sunlight than when exposed to a dull light. He also states that if glass plates covered with films of chlorophyll be kept in the dark no formaldehyde is produced, no matter whether moist carbon dioxide be present or not; further, if such plates be exposed to sunlight in an atmosphere free from carbon dioxide, a very minute quantity of formaldehyde is produced; on the other hand, the plates when exposed to the sun's rays in the presence of moist carbon dioxide give a distinct formaldehyde reaction.

From this Schryver concludes that in the presence of sunlight, water, and carbon dioxide, there is a continuous production of formaldehyde, which is continually being condensed to sugar. If this condensation does not proceed rapidly enough to remove all the formaldehyde, the excess enters into combination with the chlorophyll, and, as the free formaldehyde is used up, this compound of formaldehyde with chlorophyll decomposes, setting free the former, which is converted into sugar. There is thus in the plant a mechanism by means of which the quantity of free formaldehyde is regulated, so that at no time is the amount sufficiently large to become toxic.

*Curtius and Franzen: "Ber. deut. chem. Gesells.," 1912, **45**, 1715; Reinke: "Ber. deut. bot. Gesells.," 1883, **1**, 406; Curtius and Reinke: "Ber. deut. chem. Gesells.," 1897, **30**, 201; "Sitz. Heidelberger Akad. Wiss. Math. Nat.," 1915 Abt. A.; Pollacci: "Atti. Inst. Bot. Pavia.," 1900, **6**, 1902, **8**, 1904, **10**; Usher and Priestley: "Proc. Roy. Soc., Lond.," B., 1906, **77**, 369.

†Ewart: "Proc. Roy. Soc., Lond.," B., 1908, **80**, 30.

‡Schryver: *id.*, 1910, **82**, 226.

With a view to throwing some light on the mechanism of photosynthesis, Wager* has studied the decomposition of chlorophyll on exposure to oxygen both in the light and in the dark, with the result that he finds that the process is not catalytic. Oxygen is absorbed and aldehydes are formed, and it is suggested that the sugars produced during assimilation are not formed directly from carbon dioxide and water but by the polymerization of aldehydes produced in this way. Warner† also has found that formaldehyde is produced when chlorophyll is exposed to sunlight or electric light in air; since this substance is produced both in the presence and in the absence of carbon dioxide, it would appear that the latter plays no part in the production of formaldehyde by photosynthesis outside the plant, and that the formaldehyde is in reality an oxidation product of the chlorophyll.

The above-mentioned investigations were carried out with impure chlorophyll, Jörgensen and Kidd,‡ on the other hand, used chlorophyll *a* and *b* (see p. 227) in a state of purity which satisfied Willstätter and Stoll's criteria. They experimented with a chlorophyll sol with water as the dispersion medium. On exposing this sol, contained in glass vessels and in contact with various gases, to light, they found that formaldehyde was only produced in the presence of oxygen. In the case of contact with carbon dioxide, phæophytin (see p. 231) was produced, and there was no further change. In oxygen the chlorophyll turned yellow, due to the presence of phæophytin, and ultimately was bleached; when the bleaching is in progress, formaldehyde occurs in but small quantity, but when the bleaching is complete, there is an increase in the amount of formaldehyde. They suggest that the formaldehyde arises chiefly from the phytol which probably is split off from the chlorophyll under the action of light and oxygen.

In conclusion, mention may be made of a simple way of demonstrating the production of formaldehyde from chlorophyll, due to Osterhout.§

* Wager: "Proc. Roy. Soc.," B., 1914, 87, 386.
† Warner: *id.*, 378.
‡ Jörgensen and Kidd: *id.*, 1916, 89, 342.
§ Osterhout: "Amer. J. Bot.," 1918, 5, 511.

A solution of chlorophyll is made with carbon tetrachloride, and in it filter paper is soaked. The filter papers are dried, moistened with water, and placed on the inner surface of a glass bell-jar. The bell-jar is inverted over a dish of water, sealed from the air, and exposed to sunlight. The chlorophyll papers are gradually bleached; when pale green in colour the water in the dish gives a positive reaction for aldehyde. The same result was obtained when the carbon dioxide was excluded or increased to 10 per cent, which indicates that the aldehyde is due to the colouring matter rather than the carbon dioxide. Like results obtained when various aniline dyes, notably methyl green and iodine green, were used in place of the chlorophyll.

# SECTION III.

## THE CARBOHYDRATES.

UNDER the general heading of carbohydrates are included the sugars, starches, gums and celluloses, all of which substances play an important part in the economy of the plant. Many, such as starch, sugars, inulin and glycogen, are all-important as food materials, and are stored up for future use in various tissues. Speaking generally, the carbohydrate reserves are at a maximum in the autumn and sink to a minimum in the early summer, after the expansion and growth of the leaves and young shoots. Other carbohydrates, such as the celluloses, play an important part in the mechanics of the plant, since they are largely concerned in the formation of cell-walls; some have merely a transitory existence, such as maltose; and finally, some possibly may be degradation products—e.g., mucilages. Notwithstanding the differences in the physiological significance of the various types of carbohydrates in the plant, these substances are all closely related chemically, being composed of the same elements—carbon, hydrogen and oxygen—united together in a similar fashion.

The term carbohydrate originated through the erroneous conception that these substances were compounds of carbon with water, since the proportion of hydrogen to oxygen in all of them is the same as in water, as may be seen from the formula for grape sugar, which is $C_6H_{12}O_6$, but which might be written $C_6.6H_2O$.

### CLASSIFICATION OF CARBOHYDRATES.

On purely physical grounds such as appearance, solubility in water, taste, etc., the carbohydrates may be roughly divided into sugars and non-sugars; the systematic classification of the carbohydrates is, however, based upon their behaviour

towards hydrolytic agents, such as mineral acids or enzymes. Thus there are a considerable number of naturally occurring sugars containing five and six carbon atoms which cannot be hydrolysed; such sugars form a group known as *monosaccharides.** On the other hand many sugars are known which on hydrolysis break up into two molecules of monosaccharide according to the equation

$$C_{12}H_{22}O_{11} + H_2O = 2C_6H_{12}O_6$$

Such sugars are known as *disaccharides.*

Similarly sugars which on hydrolysis give three molecules of monosaccharide as follows—

$$C_{18}H_{32}O_{16} + 2H_2O = 3C_6H_{12}O_6$$

are termed *trisaccharides.*

Finally, carbohydrates, such as starch and cellulose, which on hydrolysis yield an unknown number of molecules of monosaccharides are classed as *polysaccharides.*

The nomenclature of the monosaccharides is based on the number of carbon atoms in their molecules, those containing five being called pentoses, while those containing six atoms are known as hexoses. For this reason the use of the terms monose and biose in place of monosaccharide and disaccharide is to be deprecated owing to the confusion which is liable to result therefrom.

I. Sugars.
- Monosaccharides.
  - Pentoses ($C_5H_{12}O_5$). Arabinose, Xylose, Rhamnose, Fucose, Quinovose.
  - Hexoses ($C_6H_{12}O_6$). Dextrose, Levulose, Sorbose, Galactose, Mannose.
- Disaccharides.
  - ($C_{12}H_{22}O_{11}$). Sucrose, Turanose, Maltose, Isomaltose, Cellobiose, Gentiobiose, Trehalose, Lactose, Melibiose.
  - ($C_{11}H_{20}O_{10}$). Glucoxylose, Primeverose, Vicianose.
- Trisaccharides ($C_{18}H_{32}O_{16}$). Raffinose, Melecitose, Gentianose.
- Tetrasaccharides ($C_{24}H_{42}O_{21}$). Stachyose.
- Unknown Constitution. Lupeose, Agavose.

* The artificially prepared tetroses, heptoses, octoses, and nonoses also belong to this group, but as they do not occur in nature, as far as is known, they need not be considered here.

II. Non-sugars or Polysaccharides.
- Starches $(C_6H_{10}O_5)n$. Starch, Dextrin, Glycogen, Inulin, Mannosanes, Galactosanes.
- Gums (*a*) Natural Gums and Pentosanes. (*b*) Mucilages and Pectic bodies.
- Celluloses $(C_6H_{10}O_5)n$.

## CONSTITUTION AND ISOMERISM OF SUGARS.

The analysis of any one of the hexose sugars, such as dextrose, levulose, galactose or mannose, would yield the same result; viz., 40 per cent of carbon, $6{\cdot}\dot{6}$ per cent of hydrogen, and $53{\cdot}\dot{3}$ per cent of oxygen; and this notwithstanding the fact that these sugars are different substances.

From the results of an analysis, it is possible to determine the simplest ratio of the atoms to each other in the molecule by dividing each percentage by the atomic weight of the corresponding element, and then determining the simplest numerical ratio between the resulting numbers:—

$$C = \frac{40{\cdot}0}{12} = 3{\cdot}\dot{3}\,;\ H = \frac{6{\cdot}\dot{6}}{1} = 6{\cdot}\dot{6}\,;\ O = \frac{53{\cdot}\dot{3}}{16} = 3{\cdot}\dot{3}$$

$$\therefore C : H : O = 3{\cdot}\dot{3} : 6{\cdot}\dot{6} : 3{\cdot}\dot{3}$$

$$= 1 : 2 : 1.$$

The formula $CH_2O$ thus arrived at, is known as the Empirical Formula; it indicates the *ratio* of the number of different atoms in the molecule, but does not indicate their actual number. The formula which, while maintaining the above ratio, also shows the actual number of atoms present in the molecule, is known as the Molecular Formula; and it can only be assigned correctly when the molecular weight is known. Now the molecular weight of all these sugars is 180, hence their molecular formula must be $(CH_2O)_6$ or $C_6H_{12}O_6$.

Compounds such as the various hexoses which have the same molecular formula and yet are not identical are said to be isomers.

The carbohydrates exhibit two kinds of isomerism, known respectively as structural and stereo-isomerism.

*Structural isomerism* is well illustrated by the two sugars dextrose and levulose. A study of their reactions, which need not here be detailed, leads to the conclusion that they both

contain five hydroxyl (OH) groups ; that dextrose belongs to the class of compounds known as aldehydes, which are characterized by the group – CHO ; and that levulose is a ketone and therefore contains the group = CO. These facts are all explained by the following constitutional formulæ :—

*Stereo-isomerism* is the second type of isomerism, and is exhibited by the three sugars dextrose, mannose and galactose, all of which are aldehydes, and have therefore the same structural formula. The possibility of isomerism in this case is accounted for by the presence in these molecules of what are known as asymmetric carbon atoms. Writing the formula for dextrose once more in a slightly different way, it will be seen that the carbon atom printed in "clarendon" (**C**) has its four valencies attached respectively to the groups ($CH_2OH.CHOH.CHOH.CHOH$)–, H–, –OH, and –CHO :—

Any carbon atom whose valency is satisfied by four different groups or elements, whatever their nature may be, is said to be asymmetric, since it is possible to represent it by either of two solid models which are not super-imposable, the one being the mirror image of the other ; there exists, therefore, between two modifications of such an asymmetric carbon atom a difference due to the different spacial distribution of the four substituting groups around it. Now the isomerism existing between glucose and mannose is accounted for by their each containing one of the two possible modifications of this same asymmetric carbon atom. Similar considerations will show

that each of the three carbon atoms marked with a star is also asymmetric, and it is therefore not surprising that it is possible to account for no less than sixteen different isomeric aldehyde sugars or aldoses; of these, however, relatively few have been found in nature.

The constitution of glucose is ordinarily represented by the formula $CH_2OH\ CHOH\ CHOH\ CHOH\ CHOH\ CHO$, which shows it to be a pentahydric alcohol and an aldehyde at the same time. When dissolved in water, however, it behaves in a peculiar manner, exhibiting the phenomenon of muta-rotation, that is to say, the optical activity of the resulting solution does not attain a steady value until some time after the solution has been made up.

The change is supposed to be connected with some alteration in its molecular configuration which may be explained by assuming that the compound

$$CH_2OH\ CHOH\ CHOH\ CHOH\ CHOH\ CH(OH)_2$$

is temporarily formed,§ but that water is thereupon split off again between one of the hydroxyl groups of the terminal carbon atom and the hydroxyl attached to the fourth carbon atom as follows:—

$$CH_2OH\ CHOH\ \overset{OH}{\overset{|}{C}}H\ CHOH\ CHOH\ CH(OH)_2 \rightarrow$$

$$CH_2OH\ CHOH\ \overset{\overset{\overset{\overset{O}{\frown}}{|}}{|}}{C}H\ CHOH\ CHOH\ \overset{|}{\underset{*}{C}}HOH + H_2O$$

It will be seen that in this formula, sometimes known as the $\gamma$ lactone or butylene oxide formula, the terminal carbon atom (marked with an asterisk) has now become asymmetric, whereas it was not so before; this method of writing the formula involves the possible existence of two optically isomeric varieties of ordinary glucose, both of which are in fact known.† When glucose is crystallized from 70 per cent alcoholic solution at ordinary temperatures, a modification known as *a*

§ Compare the formation of similar compounds from other aldehydes (p. 56).

† Tanret: "Compt. rend.," 1895, **20**, 1060; Lowry: "J. Chem. Soc.," 1899, **75**, 213; 1903, **83**, 1314.

glucose is obtained whose specific rotation is $\alpha_D = +110°$; if crystallized from water at a temperature above 98°, another variety, known as $\beta$ glucose ($\alpha_D = +19°$), is obtained; if either $\alpha$ glucose or $\beta$ glucose is dissolved in water, a gradual change in rotation is observed until a steady value of $\alpha_D = 52{\cdot}5°$ is attained, which is regarded as the specific rotation of an equilibrium mixture of $\alpha$ and $\beta$ glucose. The attainment of the stable condition is accelerated by acids, and is practically instantaneous in presence of traces of alkali.

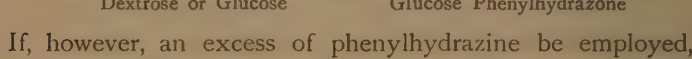
The work of Fischer and others has also brought to light the existence of yet another variety of glucose* having an $\alpha$-ethylene oxide ring structure

instead of the $\gamma$-butylene oxide, or $\gamma$ lactone, ring of ordinary glucose. So far only derivatives of $\alpha$-ethylene oxide glucose are known, the free substance not having been isolated, but there is reason to believe that its reactivity far exceeds that of the ordinary $\alpha$ or $\beta$ glucose.

## GENERAL REACTIONS OF SUGARS.

There are no reagents except that of Molisch (p. 71) which are of general application for the characterization of sugars, but there are two, namely phenylhydrazine and Fehling's solution, which react with by far the greater number of sugars and are consequently very largely used.

Phenylhydrazine, which was discovered by Fischer, reacts only with sugars containing either an aldehyde or ketone group to form, in the first place, phenylhydrazones, which in many cases are characteristic crystalline solids, but are usually soluble in water; this reaction may be illustrated thus :—




If, however, an excess of phenylhydrazine be employed, a second hydrazine complex is introduced into the compound,

* Fischer: "Ber. deut. chem. Gesells.," 1914, 47, 1980; Irvine, Fyfe, and Hogg: "J. Chem. Soc.," 1915, 107, 524.

and the resulting substance is termed an osazone. Both glucose and levulose yield the same osazone,

$$CH_2OH(CHOH)_3—C—CH : NNHC_6H_5$$
$$\quad\quad\quad

For example, in cherry or wood gum they occur in the form of pentosanes or pentosides, which substances may be regarded as anhydrides of pentoses, since they give rise to these sugars on hydrolysis in much the same way that starch on hydrolysis yields glucose.

Pentoses, although commonly looked upon as waste products, do not accumulate as the plant grows older; they fluctuate in amount according to the conditions of growth, such as the amount of available water. In the case of *Parthenium argentatum*, a high production of pentose coincides with the period of growth during which the production of rubber is at its highest. Spoehr considers that the pentoses are definite stages in the elaboration of substances such as rubber and nucleic acid.*

GENERAL PROPERTIES OF PENTOSES.

1. They are not fermentable by yeast.

2. On distillation with hydrochloric or sulphuric acid, they are converted in furfural, which may be detected by its turning a solution of aniline acetate red.

$$C_5H_{10}O_5 - 3H_2O = C_4H_3O.CHO$$

This may easily be seen by boiling some wood shavings with concentrated hydrochloric acid in a test tube and allowing the escaping steam to impinge upon a piece of filter paper moistened with aniline acetate; † a pink colour is produced.

It should be noted that hexoses‡ will also produce this reaction, though to a much smaller extent, since the quantity of furfural produced from them is much less (not more than 0·2 per cent) than in the case of the pentoses. The chief product of the action of concentrated hydrochloric acid in hexoses is levulinic acid.

3. Warmed with concentrated hydrochloric acid (sp. gr. 1·2) and a little orcinol, they produce a greenish-yellow colour which is soluble in amyl alcohol to a clear green solution having a characteristic absorption spectrum with bands between the C and D lines.

* Spoehr: "Plant World," 1917, **20**, 365.

† Prepared by mixing together equal parts of aniline, water and glacial acetic acid.

‡ Cf. Tollens: "J. f. Landw.," 1901, **49**, 39.

The reaction may be modified by adding a couple of drops of ferric chloride to the solution after it has been heated with hydrochloric acid and orcinol, when a bright green colour is produced.

*N.B.* This test is characteristic for pentoses.

4. By substituting phloroglucinol for orcinol in the above test, a red colour is produced, which changes to a brown precipitate; the latter is soluble in amyl alcohol, the solution having an absorption band between the D and E lines. This is the same reaction that is employed for the detection of lignified tissues; its use in this case depends on the fact that lignocellulose contains a pentose or furfural-yielding complex (see p. 165).

Dextrose and levulose when subjected to this test produce a yellow or brown colour.

5. Pentoses answer Molisch's test for carbohydrates. This test, which is also dependent on the formation of furfural from the sugar, consists in adding 2 c.c. of concentrated sulphuric acid to a mixture of the sugar solution with 2 drops of 15 per cent alcoholic solution of $\alpha$-naphthol, which must be free from acetone. At the junction of the two liquids a green ring is produced, and over this a red zone; on cooling and shaking the colour changes to purple.

This test is given by *all carbohydrates* and *glucosides*, and proteins which contain a carbohydrate radicle.

6. They form osazones.

7. The pentoses reduce Fehling's solution.

### PROPERTIES OF INDIVIDUAL PENTOSES.

#### *Arabinose.*

Arabinose is best obtained by the hydrolysis of cherry gum with 4 per cent sulphuric acid; it can also be obtained by the hydrolysis of gum arabic and of peach gum. Arabinose has a very sweet taste, is dextro-rotatory, $a_D$ in 10 per cent solution = + 105°, crystallizes in prisms, and melts at 160°; it reduces Fehling's solution, and yields with diphenylhydrazine a characteristic diphenylhydrazone, melting at 218°.*

* Neuberg: "Ber. deut. chem. Gesells.," 1900, 33, 2243.

### *Xylose.*

Xylose may be obtained by the hydrolysis of xylane or wood gum, and also from brewers' grains, maize, fruits, straw, and various forms of cellulose. It is optically inactive, and crystallizes in prisms, melting at 144-145°. When oxidized with bromine it gives xylonic acid, which may be identified by the fact that it forms an insoluble double salt with cadmium bromide.*

Xylose may be conveniently obtained, in about a 12 per cent yield, by boiling 1 kg. of corn cobs† (previously soaked and washed in 2 per cent ammonia solution) for 2 hours under a reflux condenser with 8 litres of 7 per cent sulphuric acid. The solution is filtered on a Buchner funnel through cloth, and is then carefully neutralized with calcium hydroxide, avoiding alkalinity. After filtering, the solution is mixed with 5 c.c. of syrupy phosphoric acid, and decolorized with charcoal; it is then mixed with twice its volume of 95 per cent alcohol, once more filtered, and evaporated in a vacuum to a thick syrup, then mixed with 95 per cent alcohol, and allowed to crystallize.

### *Methyl Pentoses* ($CH_3C_5H_9O_5$).

(*a*) *Rhamnose.*—Rhamnose, sometimes wrongly called isodulcite, has the empirical formula $C_6H_{12}O_5$, and is a pentose in which one of the hydrogen atoms has been replaced by a methyl group, its constitution being represented by the formula

$$CH_3CHOH\ CHOH\ CHOH\ CHOH\ CHO$$

In common with other methyl pentoses it yields on distillation with hydrochloric acid, methylfurfural; this latter may be detected by warming a little of the distillate with an equal volume of concentrated hydrochloric acid, when a yellow colour is produced.

Rhamnose has been obtained by the hydrolysis of a number of glucosides, e.g., quercitrin, hesperidin, and xanthorhamnin, and also saponins. The substance forms glistening crystals, m.p. 93°; $a_D = +8{\cdot}07°$, and gives a phenylosazone melting at 180°.

* Widstoe and Tollens: "Ber. deut. chem. Gesells.," 1900, 33, 136.

† Hudson and Harding: "J. Amer. Chem. Soc.," 1917, 39, 1038; 1918, 40, 1601.

(*b*) *Fucose.*—Fucose, which is isomeric with rhamnose, may be obtained by the hydrolysis of sea-weeds by means of dilute sulphuric acid; it crystallizes in microscopic needles, and yields a hydrazone, m.p. 172-173°.

(*c*) *Quinovose*, another methyl pentose isomeric with rhamnose, is produced by the hydrolysis of quinovite, a substance formed by boiling quinovin contained in the bark of *Cascarilla hexandra* with alcohol and hydrochloric acid.

### B. *HEXOSES.*

There are no convenient general reactions for distinguishing hexoses from any other group of sugars, but each of the hexoses occurring in nature are readily identified by characteristic reactions.

### GLUCOSE OR DEXTROSE.

The substance which is commonly known as grape sugar occurs, together with levulose or fruit sugar, in a number of sweet fruits, in honey, and in the seeds, leaves, roots, and blossoms of a great many of the higher plants; monosaccharides also obtain in lower plants; thus, Hunger describes them as occurring in small granules near the plastids in *Dictyota.* Glucose is formed by the hydrolysis of cane sugar, of glucosides, and of many polysaccharides, such as starch, cellulose, etc.

#### *Preparation of Glucose.*

The most convenient source for the preparation of glucose on a small scale is cane sugar. One hundred and twenty c.c. of 90 per cent alcohol mixed with 5 c.c. of fuming hydrochloric acid are heated at 45-50°; 40 grams of powdered cane sugar are now added, the mixture being kept thoroughly stirred. After two hours the solution is allowed to cool, and a little anhydrous glucose is added to induce crystallization. In the course of a few days the resulting crop of crystals is filtered off and washed with a little dilute alcohol; it is recrystallized by dissolving in half its weight of warm water and adding twice as much 90-95 per cent alcohol, filtering warm and setting aside to cool.

On a commercial scale glucose is best prepared by heating

freshly prepared potato or maize starch with dilute sulphuric acid in sealed copper vessels under 3 atmospheres pressure. When the hydrolysis is complete, the acid is removed as calcium sulphate by the addition of powdered chalk, and the filtered solution, after being decolorized by means of animal charcoal, is evaporated in a vacuum; a little anhydrous glucose in then introduced, and the syrup is allowed to crystallize at a temperature of 40°. Prepared in this way, the glucose forms a rather soft cake of small crystals; it is not a pure product, being contaminated with maltose, isomaltose (p. 86), and dextrin; it may, however, be purified by recrystallizing from aqueous alcohol.

Commercial dextrose is employed as a substitute for cane sugar for the sweetening of cheap jams, etc., but its sweetness is only about three-fifths that of cane sugar. The use of impure sulphuric acid containing arsenic for the hydrolysis of starch, and the subsequent employment of the glucose in the preparation of beer, has been the cause of the numerous deaths from arsenical poisoning.

### *Properties.*

Glucose separates from alcoholic solution or from concentrated aqueous solutions at 30-35° in needle-shaped crystals, which are anhydrous; from cold aqueous solutions, however, it crystallizes with one molecule of water ($C_6H_{12}O_6 . H_2O$) in the form of plates. It is readily soluble in water, but only very slightly soluble in absolute alcohol. It is readily fermented by yeast.

Glucose is dextro-rotatory, $\alpha_D = 52{\cdot}5°$; it is sometimes known as dextrose to distinguish it from the lævo-rotatory sugar levulose with which it is frequently found associated in ripe fruits.

### *Reactions.*

1. In the presence of ammonia, glucose can reduce silver from its salts. A little glucose is added to a solution of silver nitrate to which have been added a few drops of caustic potash and just sufficient ammonia to redissolve the brown precipitate. On warming the mixture the silver is deposited on the sides of the test tube, forming a mirror.

2. Nylander's Test.—When boiled with a solution of glucose Nylander's reagent turns brown and finally black owing to the precipitation of bismuth oxide and metallic bismuth.

The reagent is prepared by dissolving 2 grams of bismuth oxynitrate and 4 grams of Rochelle salt in 100 grams of 10 per cent caustic soda solution.

3. Add to the solution basic lead acetate and ammonia. If glucose be present, a white precipitate comes down, which turns red. This reaction is not given by cane sugar.

4. Add to the solution a little copper sulphate solution and an excess of caustic potash. On warming, a yellow to red precipitate is formed. This reaction also is given by levulose and maltose, but not by saccharose.

5. On warming with Fehling's solution, a red precipitate is given by dextrose, levulose and maltose, but not by saccharose.

6. Add a little Barfoed's reagent and warm. A red precipitate floating as a thin film on the surface of the liquid indicates dextrose. This reaction is also given by levulose but not by cane sugar or maltose.

The reagent, which should be freshly made up, is prepared by dissolving 6·5 grams of copper acetate in 100 c.c. of water containing 1 gram of glacial acetic acid.

7. The addition to the solution of picric acid and caustic soda results in the formation of a blood-red coloration, due to picramic acid. This reaction is also given by other sugars.

8. On boiling the solution of glucose with an equal volume of caustic potash, a yellow-brown colour results; on acidifying with dilute nitric acid the colour lightens and a smell of burnt sugar is produced.

9. Glucose reacts with phenylhydrazine to give an osazone. To 5 c.c. of an approximately 5 per cent solution of glucose, add 4 or 5 drops of phenylhydrazine and about the same amount of glacial acetic acid. (If phenylhydrazine hydrochloride is used, add about enough solid to cover a threepenny piece and an equal quantity of sodium acetate.) Place the mixture in a boiling water bath for about half an hour and then remove; a golden yellow crystalline precipitate will have been formed. On examination under the microscope the needle-shaped crystals will be seen to be gathered together

in clusters resembling wheat sheaves. Glucosazone melts at 204-205° with decomposition; it is insoluble in water but soluble in alcohol, the solution being lævo-rotatory in contradistinction to that of maltose which is dextro-rotatory.

### *Microchemical Tests.*

For microchemical tests for sugars, the reduction of copper salts in the presence of excess of alkali is generally employed, but these are not altogether satisfactory, owing to the amount of diffusion which takes place, and also because sucrose, if its presence in a tissue be suspected, must first be hydrolysed by boiling with acid before the reduction will take place.

Mangham * and others have obtained excellent results by the use of the osazone test for microscopic work; if properly performed, it is much more satisfactory than any other, and has the advantage of being a very delicate test for some sugars. For example, a ·015 per cent solution of glucose will give a definite reaction. The main disadvantage of the method is in its comparative slowness.

Two solutions are required:

(*a*) 1 gram of phenylhydrazine hydrochloride dissolved in 10 grams of glycerine.

(*b*) 1 gram of sodium acetate dissolved in 10 grams of glycerine.

If necessary the solution of these substances may be hastened by means of heat, and before use the solutions should be filtered.

Glycerine is used because its penetrative power is greater than that of water, and also because it will not evaporate and deposit crystals of the substances used.

For use, one drop of each fluid is placed on a glass slip and mixed thoroughly. The section, which must be more than one cell in thickness, is laid in the mixture and covered with a cover glass. The preparation is heated on a hot water oven for about half an hour, and is then allowed to cool; the osazone crystals will form in varying degrees of rapidity.

In order that familiarity with the method may be gained, the reagents may be mixed on the slip with drops of sugar

* Mangham: "New Phytol.," 1911, 10, 160; "Ann. Bot.," 1915, 29, 360.

solution of different concentrations heated for varying periods and examined periodically after cooling.

Maltose gives an osazone characterized by dense rosettes of lemon yellow crystals, which are broader and larger than those obtained with dextrose and levulose; the crystals may, however, take several weeks for their formation.

Dextrose and levulose may be distinguished by the fact that methylphenylhydrazine gives a crystalline osazone with levulose and not with dextrose. It is to be noted that the methylphenylhydrazine must be very pure.

### LEVULOSE OR FRUCTOSE.

Levulose occurs in most sweet fruits and in honey, together with both cane sugar and dextrose, but usually in excess of the latter two. It is formed in equal quantity with dextrose by the hydrolysis of cane sugar, but being more strongly lævorotatory than dextrose is dextro-rotatory, the resulting mixture turns the plane of polarized light to the left, whereas the original cane sugar is dextro-rotatory; the resulting mixture is accordingly known as invert sugar and the process by which this change is produced is called inversion.

The separation of pure levulose from invert sugar on a small scale is not easy to carry out, but the operation is performed on a large scale by making use of the fact that on treating invert sugar with milk of lime the levulose is converted into an insoluble calcium compound, which may be filtered off and purified, while the glucose remains in solution. The easiest means of preparing levulose in the laboratory is to hydrolyse inulin by boiling 1 part of this substance with 5 parts of ·5 per cent sulphuric acid * for one hour; the acid is then removed by means of barium carbonate, and the solution, after being treated with animal charcoal and filtered, is evaporated at a low temperature to a thin syrup. The latter is then crystallized from alcohol after sowing with a crystal of pure levulose. A modification of this method is employed for the manufacture of pure levulose. †

* Düll ("Chem. Zeit.," 1895, 19, 216) recommends the use of oxalic acid; see also Wiechmann: "Z. d. Vereins Deut. Zuckerind.," 1891, 41, 331.

† Cf. Stein: "Proc. Internat. Confer. Sugar Ind.," April, 1908.

*Properties.*

Levulose separates from alcohol in hard rhombic crystals, which have the composition $C_6H_{12}O_6$; from concentrated aqueous solutions, however, it crystallizes in needles with water of crystallization $2C_6H_{12}O_6 . H_2O$. It is fairly soluble in hot absolute alcohol and ether, and may thus be separated from other sugars which are insoluble in these solvents. Levulose is strongly lævo-rotatory and exhibits slight muta-rotation; its rotatory power is very dependent on temperature, $a_D^{20} = -93$ in a 10 per cent solution.

*Reactions.*

1. To a solution of levulose mixed with an equal volume of concentrated hydrochloric acid a few grains of resorcin are added. On warming, a deep red coloration results, and finally a brown-red precipitate. The precipitate is soluble in alcohol, giving a deep red solution.

This reaction is given by all keto-hexoses and by carbohydrates such as cane sugar and raffinose which give rise to them on hydrolysis.

2. Levulose gives the same reactions as dextrose with salts of copper and picric acid.

3. Levulose with milk of lime forms an insoluble compound; dextrose does not.

4. Levulose gives with phenylhydrazine the same osazone as glucose, namely glucosazone.

5. With methylphenylhydrazine it gives, in alcoholic solution, an osazone crystallizing in needles; m.p. 158°. (Distinction from glucose.)

SORBOSE.

Sorbose is a ketonic sugar produced by the fermentative oxidation of the alcohol sorbite contained in the sap of the mountain ash, *Pyrus Aucuparia*; this sugar probably does not exist as such in the plant, but is produced by oxidation as described.

GALACTOSE.

This sugar is formed as a product of the hydrolysis primarily of milk sugar, but also of the gums occurring in

peaches and plums, and from the so-called pectic substances occurring in carrots and pears; in all these cases it is accompanied by other sugars, which may be either hexoses or pentoses. Galactose also occurs in several plants belonging to the Caryophyllaceae. It is also formed by the hydrolysis of the trisaccharide raffinose, of the glucoside digitalin, and of a glucoside occurring in the ivy.

### *Preparation.*

Galactose is best prepared by boiling milk sugar for six hours with four times its weight of 2 per cent sulphuric acid; the solution is then evaporated, and a few crystals of galactose are added to induce crystallization. After some time the crude galactose crystallizes out; it is purified by dissolving in four-fifths of its weight of water, and mixing the resulting solution with twice its volume of 93 per cent alcohol; the precipitate is filtered off and dried.

### *Properties.*

Galactose crystallizes in minute hexagonal crystals, which melt at 168°. It is strongly dextro-rotatory, $a_D = 83{\cdot}8°$, and exhibits muta-rotation; it ferments completely, but rather more slowly than glucose.

### *Detection.*

1. The hexagonal form of the crystals is characteristic of galactose.

2. It gives a phenylhydrazone (m.p. 158-160°) which is lævo-rotatory.

3. It reduces Fehling's solution somewhat more slowly than glucose; 1 c.c. Fehling's solution $\equiv$ 5·11 mg. galactose.

4. On oxidation with nitric acid it yields mucic acid. Five grams of substance are heated in a beaker with 6 c.c. of nitric acid (sp. gr. 1·15) until two-thirds of the liquid have been evaporated off. After twelve hours the mucic acid formed will have separated, and may be washed with 10 c.c. of water. If other insoluble substances, such as cellulose, etc., are present, place the filter paper with the solid in a dilute solution of ammonium carbonate to extract the mucic acid as ammonium

salt. Filter once more, and evaporate the filtrate almost to dryness, and acidify with nitric acid; the precipitate is pure mucic acid.

### MANNOSE.

Mannose may be obtained by the hydrolysis of a form of mannane contained in salep mucilage (*Orchis Morio*) and from several other so-called hemi-celluloses contained in peas, coffee beans, date stones, etc. It is most conveniently prepared by the hydrolysis of the hemi-cellulose contained in ivory nuts, the fruits of *Phytelephas macrocarpa;* turnings from these seeds, obtained in the manufacture of vegetable ivory buttons, are ground up and 150 gms. are added in portions to 150 gms. of 75 per cent sulphuric acid, keeping the temperature below 40° C.* After standing for some hours the mixture is diluted to two litres and gently boiled under a reflux condenser for three hours. After carefully neutralizing with slaked lime, the solution is filtered and decolorized by means of charcoal and then by lead acetate followed by hydrogen sulphide; it is then evaporated in a vacuum to a syrupy consistency and mixed with an equal volume of glacial acetic acid and allowed to crystallize.

Mannose has a sweet taste followed by a bitter one; when dry, it is a hard crumbling substance, which, however, deliquesces and is readily soluble in water; it is only slightly soluble in hot alcohol and is insoluble in ether. It is dextrorotatory, $[\alpha]_D^{20} = +14{\cdot}36^\circ$ in 10 per cent solution, and is readily fermentable by yeast.

#### *Detection.*

1. Mannose is most readily detected and estimated by means of its phenylhydrazone, which is almost insoluble in water, and forms almost at once on adding phenylhydrazine acetate to an aqueous solution of the sugar; the phenylhydrazone is soluble in a very large volume of boiling water, and separates in fine prisms from the solution on cooling. These crystals melt at 195-200°.

* Hudson and Sawyer: "J. Amer. Chem. Soc.," 1917, 39, 470.

An excess of phenylhydrazine converts mannose into glucosazone, which is identical with the substance obtained under similar conditions from both glucose and levulose.

2. Mannose reduces Fehling's solution, 10 c.c. = ·4307 gm. mannose.

Both mannose and galactose are sometimes considered to be transitory substances; an idea supported by the fact, observed by Hérissey,* that seminase is found in the seeds of lucerne; and that during germination cane sugar is relatively abundant, while mannose and galactose are not found, at any rate in any quantity.

## DISACCHARIDES.

### CANE SUGAR, SUCROSE OR SACCHAROSE.

Cane sugar is one of the most widely distributed substances to be found in the vegetable kingdom. Besides forming about 20 per cent of the juice of the sugar cane, *Saccharum officinarum*, and about 10 to 20 per cent of that of the beetroot, it is found in varying quantities in the wood of maple and birch, and in *Sorghum saccharatum;* it occurs, moreover, in wheat, maize, barley, in carrots and in madder root. In most sweet fruits it is found together with a greater or lesser quantity of dextrose and levulose, which may possibly have been formed from it by hydrolysis. It also is found in the leaves of many plants associated with glucose and maltose. The following table, compiled by Kulisch, gives the relative proportions of cane sugar and hexoses found in various fruits.

| | Cane Sugar. | Hexoses. |
|---|---|---|
| Pine apple | 11·33 | 1·98 |
| Strawberry | 6·33 | 4·98 |
| Apricot | 6·04 | 2·74 |
| Ripe banana | 5·00 | 10·00 |
| Apple | 1-5·40 | 7-13·00 |

In honey practically only invert sugar † is found, although the sugar found in the flowers by the bees is commonly cane sugar. The hydrolytic agent in this case is, however, most probably the formic acid secreted by the bees.

The two chief sources for the preparation of cane sugar on

* Hérissey: "Rev. gen. Bot.," 1903, **15**, 345, 369, 406, 444.

† A mixture of dextrose and levulose, see p. 64.

a manufacturing scale are the sugar cane and the beet. The processes used in both cases are more or less similar, and consist in obtaining the juice, purifying it, concentrating it and, lastly, crystallizing it. The juice is generally obtained from the cane by crushing, as much as 85-95 per cent of the juice being expressed in this way; in some cases it is extracted by diffusion, which consists in immersing the cane in water, when the sugar diffuses out of the cells into the surrounding water while the indiffusible colloids remain behind. The crude juice is then boiled with milk of lime, in order to neutralize any acid present and to precipitate coagulable proteins, and is subsequently treated with carbon dioxide. After filtering, the solution is concentrated in a vacuum and allowed to crystallize, the mother liquor being separated by centrifugalizing; the crystals may be used at once as brown sugar, or may be refined.

When the beet is used, the roots are first cut into slices and subjected to diffusion, the same quantity of water circulating through a series of vessels in such a manner that the fresh water first passes over material from which most of the sugar has already been extracted, and as the solution becomes more concentrated, it comes into contact with material which is increasingly richer in sugar. In this way the aqueous extract attains a concentration of from 12-15 per cent.* This solution is then boiled with lime and saturated with carbon dioxide to decompose any calcium saccharosate which may have been formed; it is then filtered and again saturated with carbon dioxide or a mixture of this gas and sulphur dioxide to precipitate the last traces of calcium, and also to decolorize it; the older process of filtration through animal charcoal is thereby rendered unnecessary; the solution is then boiled and filtered and the clear filtrate is concentrated in a vacuum and allowed to crystallize. The uncrystallizable residue which remains is known as molasses; a further yield of sugar may be obtained from this residue by the addition of lime to the cold solution or of strontia to the boiling solution whereby the cane sugar in the molasses is converted into the insoluble calcium or strontium saccharosate, which may be filtered off and decomposed by a current of carbon dioxide into cane sugar and

* The residue remaining after the extraction of the sugar is employed for cattle food.

calcium or strontium carbonate. The molasses are sometimes fermented for the manufacture of rum or may be used for cattle food; they are also used in the manufacture of boot blacking.

By suitable methods of cultivation, seed selection and use of nitrogenous and potash fertilizers the amount of sugar contained in the beet has been raised from 10·6 per cent in the period 1880-90 to about 15 per cent in the period 1900-10, and the beetroot is gradually displacing the sugar cane as a source of sucrose. Owing to exceptionally favourable weather conditions the yield in the year 1908-9 rose to about 18·5 per cent, but individual beets have been known to contain up to 27 per cent of sugar.

*Properties.*

Cane sugar crystallizes from water in monoclinic crystals which do not contain water of crystallization; it is readily soluble in water and only slightly soluble in alcohol; it is dextrorotatory, its specific rotation being $a_D = +66·5$.

When heated to 160° it melts to a glassy mass known as barley sugar, which gradually becomes crystalline again; if heated to 190-200° it is converted into an uncrystallizable brown substance known as caramel, which is used for colouring beer and wine.

*Reactions.*

1. Solutions of cane sugar heated with concentrated hydrochloric acid turn reddish pink.

2. If warmed with concentrated hydrochloric acid and a few crystals of resorcin a deep red colour is produced owing to the liberation of levulose.

3. Cane sugar does not react with phenylhydrazine.

4. Cane sugar does not reduce Nylander's reagent.

5. Solutions of cane sugar do not reduce Fehling's solution until they have been inverted by boiling for a short time with a few drops of dilute sulphuric acid; if then made alkaline and boiled with Fehling's solution reduction ensues.

If a solution in water is boiled with a few drops of mineral acid, the sign of the optical activity of the solution changes from + to −. This change, which is known as *inversion*, is due to the fact that the mineral acid hydrolyses the cane sugar,

converting it into equal molecular proportions of the two sugars dextrose and levulose,

$$C_{12}H_{22}O_{11} + H_2O = C_6H_{12}O_6 + C_6H_{12}O_6$$

and since the optical activity of levulose is greater than that of dextrose the resulting invert sugar is lævo-rotatory.

Numerous experiments have been carried out with a view to determining the conditions which bring about this inversion. Aqueous solutions of cane sugar, if kept for some time, gradually become inverted, the change being somewhat accelerated by prolonged boiling.

Similarly cane sugar solutions when heated with acids undergo inversion, the rate at which the change takes place being a measure of the strength, or better, the chemical affinity, of the acid. Extremely small quantities of acid suffice to effect the change in a boiling solution; thus 80 parts of cane sugar dissolved in 20 parts of water are completely hydrolysed by heating in boiling water for one hour with an amount of hydrochloric acid corresponding to 0·005 per cent of the weight of the sugar; within certain limits, however, the action is accelerated by increasing the concentration of the acid. If, however, the acid is too strong and the heating be continued too long, the solution is liable to darken and decompose. Moreover, prolonged action, even at temperatures of 10-15°, of concentrated acids was found by Wohl* and by Fischer† to produce exactly the opposite phenomenon, known as reversion, by which the simple molecules, more especially those of levulose, are made to condense together to form complex dextrin-like substances, as well as a disaccharide isomaltose.

6. Sucrose is not directly fermentable by pure yeast.

### TURANOSE. $C_{12}H_{22}O_{11}$.

This is a disaccharide formed by the partial hydrolysis of the trisaccharide melecitose (see p. 91); it reduces Fehling's solution, and on hydrolysis yields glucose and levulose; it is therefore isomeric with sucrose.

* Wohl: "Ber. deut. chem. Gesells.," 1890, **23**, 2092.
† Fischer: *id.*, 1890, **23**, 3687.

## MALTOSE. $C_{12}H_{22}O_{11}$.

This disaccharide has not such a wide distribution in the plant as has cane sugar. It occurs in the cell sap of leaves and is formed, at any rate in part, by the action of diastase on the starch. Maltose is produced in quantity during the germination of barley and other grains by a similar enzyme action. The action is hydrolytic, and may be represented approximately by the following formulæ :—

$$\underset{\text{Starch}}{(C_6H_{10}O_5)n} + H_2O \rightarrow \underset{\text{Maltose}}{C_{12}H_{22}O_{11}} + \underset{\text{Dextrin}}{(C_6H_{10}O_5)n}$$

The same change can also be brought about by the careful hydrolysis of starch with sulphuric acid.

Maltose is also formed by the action of diastase and other enzymes on glycogen.

In preparing maltose from starch, it is not necessary always to act on the starch contained in barley, potato-starch serving equally well, the diastase which is employed is usually introduced in the form of malt, which is barley that has been allowed to sprout and is then killed by suddenly heating to a temperature sufficient to stop the further growth of the barley without destroying the diastase. The malt is then stirred up with starch and water, and kept at a temperature of 60-62° for about half an hour ; by the end of this time about 80 per cent of the starch has been converted into maltose and 20 per cent into dextrin. Dextrin itself is also converted into maltose by diastase, but the reaction is very slow, and in practice sufficient time is not allowed to effect this change.

### *Properties and Reactions.*

Maltose is readily soluble in water, and crystallizes from this solvent in slender white needles, having the composition $C_{12}H_{22}O_{11}$, $H_2O$.

1. Maltose reduces Nylander's reagent, but not Barfoed's reagent.

2. Maltose reduces Fehling's solution without previous hydrolysis, and can therefore be estimated directly by this means.

3. When treated with phenylhydrazine, as described under glucose, it gives an osazone (m.p. 206°), which is soluble in

seventy-five parts of boiling water, and can be crystallized from this solvent in rosettes of plates or broad needles resembling sword blades; alcoholic solutions of maltosazone are dextro-rotatory. (Distinction from glucosazone.)

4. On hydrolysis, by boiling with dilute sulphuric acid, maltose breaks up into two molecules of glucose:—

$$C_{12}H_{22}O_{11} + H_2O = 2C_6H_{12}O_6$$

the rotatory power of the solution being thereby diminished.

5. The aqueous solution is strongly dextro-rotatory: $a_D = +137^\circ$; freshly-made solutions exhibit a higher rotation than older ones, owing to a negative muta-rotation.

6. Unlike cane sugar, maltose is said to be directly fermentable by yeast; this statement is, however, probably not strictly true, since pure cultures of yeast containing only zymase, the active enzyme which produces alcoholic fermentation, have no action on maltose. Ordinary brewers' yeast, however, contains maltase, which first hydrolyses maltose to grape sugar, which is then fermented by zymase. Only sugars containing six carbon atoms are fermentable by yeast (see p. 378).

### ISO-MALTOSE. $C_{12}H_{22}O_{11}$.

Iso-maltose is a disaccharide which is isomeric with and closely related to ordinary maltose, its optical activity, $a_D = +139\text{-}140^\circ$, being almost the same as that of maltose; it is formed, together with ordinary maltose, when diastase acts on starch, provided the enzyme is not present in too large a quantity; the most favourable temperature for its production is 65-70°. Iso-maltose is also formed together with dextrin by the action of concentrated hydrochloric acid on glucose at a temperature of 10-15°, which accounts for the fact that it is not infrequently met with as an impurity in commercial glucose prepared by the action of hydrochloric acid on starch (see p. 74).

### CELLOBIOSE. $C_{12}H_{22}O_{11}$.

This is a disaccharide obtained from cellulose by the action of glacial acetic acid and acetic anhydride in the presence of concentrated sulphuric acid. The resulting acetyl derivative,

on treatment with alcoholic potash, yields cellobiose. It reduces Fehling's solution and yields two molecules of glucose on hydrolysis, and is thus isomeric with maltose.

### GENTIOBIOSE. $C_{12}H_{22}O_{11}$.

This disaccharide * is obtained by the partial hydrolysis of the trisaccharide gentianose (see p. 91); by the action of emulsin it is converted into two molecules of glucose from which it follows that gentiobiose is a $\beta$ glucoside. It has been synthesized by the action of emulsin on glucose.†

### TREHALOSE. $C_{12}H_{22}O_{11}$.

Trehalose is a disaccharide found in various agarics, notably *Boletus edulis*, in moulds such as *Aspergillus niger*, and in *Selaginella lepidophylla*.‡ It does not reduce Fehling's solution and is strongly dextro-rotatory, $\alpha^{D} = +199^{\circ}$. When boiled in acids it is slowly converted into two molecules of glucose.§

### LACTOSE OR MILK SUGAR. $C_{12}H_{22}O_{11}$.

This disaccharide, though of considerable importance in the animal kingdom, is never found in plants. It reduces Fehling's solution and on hydrolysis, by the enzyme lactase or by dilute mineral acids, it yields molecular proportions of glucose and galactose.

### MELIBIOSE. $C_{12}H_{22}O_{11}$.

This disaccharide‖ is produced by the partial hydrolysis of the trisaccharide raffinose. It yields on hydrolysis molecular proportions of glucose and galactose. Melibiose is the first natural saccharide that was artificially synthesized.¶

* Zemplen: "Ber. deut. chem. Gesells," 1915, 48, 233.

† Hérissey, Bourquelot and Coirre: "J. Pharm. Chim," 1913, 7, 181.

‡ Anselmino and Gilg: "Pharm. Zeit.," 1913, 58, 563; Lippmann: "Ber. deut. chem. Gesells," 1912, 45, 3431.

§ Winterstein: "Z. physiol. Chem.," 1894, 19, 70.

‖ Scheibler and Mittelmeier: "Ber. deut. chem. Gesells," 1890, 23, 1438.

¶ Fischer and Armstrong: *id.*, 1902, 35, 3144.

## DISACCHARIDES PRODUCED BY THE UNION OF A HEXOSE WITH A PENTOSE.

Of late years several disaccharides have been discovered which on hydrolysis yield one molecule each of a hexose and a pentose; such are the two glucoxyloses isolated in the form of their dibenzoyl derivatives from a bitter principle contained in *Daviesia latifolia.**

Three other glucoxyloses are given below:—

### PRIMEVEROSE. $C_{11}H_{20}O_{10}$.

Primeverose is prepared from the glucosides primeverin and primulaverin occurring in *Primula officinalis.*† It has a free aldehyde group and would therefore appear to have the constitution,

$$CHO.[CHOH]_4.CH_2.O.CH.CH.[CHOH]_2.CH_2OH$$
$$\diagdown\diagup$$
$$O$$

### VICIANOSE. $C_{11}H_{20}O_{10}$.

This disaccharide is obtained by the hydrolysis of the glucoside vicianin occurring in *Vicia angustifolia*, and is found to be composed of one molecule of glucose and one of arabinose.‡

### STROPHANTHOBIOSE. $C_{12}H_{22}O_{10}$.

This disaccharide likewise occurs in a glucoside, strophanthin. On hydrolysis it yields mannose and rhamnose ($C_6H_{12}O_5$).§

## TRISACCHARIDES.

### RAFFINOSE.

This sugar occurs in cotton seeds, barley, eucalyptus, manna, jute, and in the beetroot; the juice of this latter contains on an average about 15 per cent of cane sugar but only 0·02 per cent‖ of raffinose. The molasses from beet sugar re-

* Power and Salway: "J. Chem. Soc.," 1914, 1069.
† Goris and Vischniac: "Compt. rend.," 1919, **169**, 871, 975.
‡ Bertrand and Weisweiller: *id.*, 1910, **151**, 325, 884.
§ Feist: "Ber. deut. chem. Gesells," 1900, **33**, 2091.
‖ Strohmer: "Oest. Ung. Z. f. Zuckerind. u. Landw.," 1910, **39**, 649.

fineries, however, contain from 2-3 per cent of raffinose (hence the name) and form the chief commercial source of this sugar.

As the concentration of the raffinose increases it tends to crystallize out together with the cane sugar in the form of mixed crystals having a peculiar and characteristic pointed appearance quite different from ordinary cane sugar.

Numerous methods * have been described for preparing pure raffinose from molasses, but as they are mostly rather tedious they will not be detailed here.

According to Bau,† raffinose may be extracted from cotton seeds by the following simple process. The powdered seeds, after being freed from fat by means of ether, are extracted with hot 70 per cent alcohol and the extract is heated with animal charcoal, filtered, and evaporated; on cooling raffinose crystallizes out and may be further purified by recrystallization from alcohol.

Raffinose crystallizes with 5 molecules of water in clusters of slender glistening needles or prisms whose composition is expressed by the formula $C_{18}H_{32}O_{16} \cdot 5H_2O$. It dissolves in water and in methyl alcohol, in which latter solvent cane sugar is only sparingly soluble, but is hardly soluble in ethyl alcohol, whereas cane sugar is appreciably soluble.

It is strongly dextro-rotatory, $\alpha_D = +104{\cdot}4°$, in 10 per cent solution, and consequently cane sugar in which raffinose occurs as an impurity appears to contain more than 100 per cent of sucrose when estimated polarimetrically; hence raffinose is sometimes known as "plus sugar".

It does not reduce Fehling's solution, nor does it react with phenylhydrazine.

On careful hydrolysis raffinose breaks up at first into levulose and a disaccharide—melibiose.

$$\underset{\text{Raffinose}}{C_{18}H_{32}O_{16}} + H_2O = \underset{\text{Levulose}}{C_6H_{12}O_6} + \underset{\text{Melibiose}}{C_{12}H_{22}O_{11}}$$

On heating further the melibiose itself is broken up as follows:—

$$\underset{\text{Melibiose}}{C_{12}H_{22}O_{11}} + H_2O = \underset{\text{Dextrose}}{C_6H_{12}O_6} + \underset{\text{Galactose}}{C_6H_{12}O_6}$$

* v. Lippmann: "Die Chemie d. Zuckerarten," 3rd ed., Braunschweig, Vol. II., p. 1628.

† Bau: "Chem. Zeit.," 1894, 18, 1796.

If boiled with mineral acid, therefore, raffinose gives rise to a mixture of dextrose, levulose and galactose.

According to Neuberg,* raffinose is hydrolysed by emulsin into cane sugar and galactose. (See below.)

Raffinose, unlike cane sugar, is completely fermented by bottom fermentation yeast to alcohol and carbon dioxide, whereas top fermentation yeast is only able to ferment it partially, converting the levulose complex into carbon dioxide and alcohol and leaving melibiose unattacked. These facts have been made use of by Bau† for detecting and for estimating raffinose.

*Detection.*

There are no rapidly performed characteristic tests for raffinose.

The only really reliable method of identifying it is to isolate the substance by precipitating the strontium compound in alcoholic solution, filtering off the precipitate and decomposing it by a current of carbon dioxide. The resulting solution is then evaporated and the residue extracted with alcohol to remove sucrose and other sugars which are more soluble in alcohol than raffinose. The pure substance should be identified by its crystalline form and optical properties.

Another way of identifying raffinose‡ is to add to the solution a little decoction of fresh yeast, to act as nutriment, and then to sterilize the solution; a pure culture of top fermentation yeast is then added to the solution and the fermentation is allowed to proceed in a thermostat at 31°; when it is completed, the solution is boiled with animal charcoal, filtered, and evaporated to a syrup; the latter is then, while still hot, poured into hot alcohol and on cooling it is filtered; the filtrate is then precipitated by mixing with 1½ vols. of ether. After 24 hours the supernatant liquid is poured off and the residual syrup, which consists of melibiose, is converted into its osazone which is characterized by its crystalline form and melting point 178-9°.§

Finally, Neuberg‖ has proposed making use of emulsin for the identification of raffinose.

* Neuberg: "Bioch. Zeitschr.," 1907, **3**, 519.
† Bau: "Chem. Zeit.," 1894, **18**, 1797; 1897, **21**, 185; 1902, **26**, 69.
‡ *Ibid.*, 1897, **21**, 185. § *Ibid.*, 1902, **26**, 69.
‖ Neuberg: "Bioch. Zeitsch.," 1907, **3**, 519 and 535.

### MELECITOSE. $C_{18}H_{32}O_{16}$, $2H_2O$.

This is a sugar which occurs in the sap of *Larix europaea* and in Persian manna; it crystallizes with two molecules of water in rhombic prisms, and is dextro-rotatory ($a_D = +83°$). It does not reduce Fehling's solution, and on hydrolysis yields first a molecule of glucose and a disaccharide—turanose, $C_{12}H_{22}O_{11}$—which subsequently itself breaks up into one molecule of glucose and one of fructose.

### STACHYOSE. $C_{24}H_{42}O_{21}$, $4H_2O$.

This substance occurs in the tubers of *Stachys tuberifera* and in a large number of leguminous seeds.* It forms plate-like crystals, which dissolve readily in water to give a faintly sweet solution, which is dextro-rotatory ($a_D = +148°$). It does not reduce Fehling's solution. When boiled with dilute mineral acid it yields one molecule each of glucose and levulose, and two molecules of galactose.†

### GENTIANOSE. $C_{18}H_{32}O_{16}$.

This trisaccharide occurs in the roots of *Gentiana lutea*. On hydrolysis by mineral acids it is converted into two molecules of glucose and one of fructose. Hydrolysis by means of dilute acids breaks it up into one molecule of fructose and one of gentiobiose (see p. 87), while *Aspergillus niger* resolves it into one molecule of glucose and one of sucrose. Gentianose does not reduce Fehling's solution.

### SUGARS OF UNKNOWN MOLECULAR WEIGHT OR SUGAR-LIKE POLYSACCHARIDES.

Of these sugars lupeose and agavose are examples. The former, which occurs in lupin seeds, does not reduce Fehling's solution, and on hydrolysis yields galactose, fructose and glucose. It is supposed to be a tetrasaccharide.‡

Agavose, obtained from *Agave americona*, is an optically inactive sugar of unknown constitution which reduces Fehling's solution.§

* Tanret; "Compt. rend.," 1912, **155**, 1526.
† Planta and Schulze: "Ber. deut. chem. Gesells," 1891, **24**, 2705.
‡ Schulze: *id.*, 1910, **43**, 2233.
§ Michaud and Tristan: "Amer. Chem. J.," 1892, **14**, 548.

## ESTIMATION OF SUGARS.

### A. *VOLUMETRIC METHODS.*

#### I. ESTIMATION BY MEANS OF FEHLING'S SOLUTION.

The principle of this method lies in the fact that certain sugars are capable of reducing copper sulphate in hot alkaline solutions to cuprous oxide, the presence of which is indicated by a yellow-red precipitate.

Fehling's solution is made up in two solutions :—

A, containing 69·28 grams of pure crystallized copper sulphate in one litre of distilled water.

B, containing 350 grams of Rochelle salt and 100 grams of caustic soda in one litre of distilled water.

The solution A must be made up very accurately, whereas the quantities required for solution B need only be roughly weighed.

For use, 5 c.c. of A are mixed with 5 c.c. of B ; the mixture is a deep blue colour, and is known as Fehling's solution. If correctly compounded, 10 c.c. of the solution contain ·11 gram of cupric oxide, which is able to oxidize ·05 gram of glucose.

This value is sufficiently correct for general purposes ; it is, however, an approximation, and varies for different sugars, the factor for levulose, for instance, is ·05144, whilst that for invert sugar is ·04941. If it be desired to obtain very accurate results, it is better to standardize the solution by titrating 10 c.c. with a solution of glucose of known strength. Such a solution may be obtained by dissolving ·95 gram of pure crystallized cane sugar in 500 c.c. of distilled water and boiling for fifteen to twenty minutes with 2 c.c. of concentrated hydrochloric acid. The solution must then be neutralized by the addition of solid sodium carbonate, and made up to 1 litre ; 50 c.c. of this solution contain ·05 gram of glucose, and should reduce exactly 10 c.c. of Fehling's solution.

It frequently happens in titrating liquid extracts from plants, etc., that the cuprous oxide will not settle down, but remains suspended in the solution as a fine turbidity. In such cases the addition of a few drops of aluminium sulphate may

sometimes cause the precipitate to subside; if not, it will be necessary to boil a fresh portion of the original solution and then to add lead acetate; after filtering, the filtrate is saturated with hydrogen sulphide to remove excess of lead, and the titration is then carried out on the filtrate after boiling off the hydrogen sulphide.

Plant extracts may also contain tannins which must be removed before estimating the sugars. There are various ways of doing this; and in all cases it is best to try the separation with a small portion of the material first, in order to determine the efficiency of the method.

1. The aqueous solution is mixed with about half its volume of ethyl acetate, and thoroughly shaken. The mixture is then placed in a separating funnel until the two solvents have separated, when the lower aqueous part, containing the sugar, is drawn off, and with it the process is repeated. The aqueous solution is now warmed on a water bath until all the ethyl acetate is driven off, and the cooled solution tested for tannin.

2. To every 100 c.c. of the solution add 4 grams of pure lead carbonate. Shake thoroughly at intervals for about four hours. Test the filtrate for tannins.

The chief objection to this method is that lead salts soluble in water may be formed.

3. Add gelatine solution until no more precipitate is formed. Filter and wash the precipitate very thoroughly. The filtrates resulting from any of these operations may then be examined for the sugar.

### *Estimation of Pentoses.*

When pentoses alone are present, they may be estimated in the same way as glucose; if, however, they are mixed with other carbohydrates, or are present in the form of pentosanes other methods must be employed (see p. 101).

### *Estimation of Glucose.*

The following precautions must be taken in estimating glucose by this and similar methods involving the reduction of metallic salts.*

* For the estimation of levulose in the presence of glucose see Loewe: "Proc. Soc. Exp. Biol. Med.," 1916, 13, 71.

1. Any substances such as tannins which may have the power of reducing the salts used in titration must be removed.

2. The strength of the sugar solution must be weak, because the reducing power of sugar varies with the concentration, hence it is best to titrate a solution of about the same strength as that used for the standardizing of the Fehling's solution. This necessitates preliminary estimation; should the strength of the solution be much above this point, add a known volume of water until the strength approximates ·5 per cent.

The titration, which should be completed as rapidly as possible in order to avoid reoxidation of the solution by the air, is performed as follows:—

Five c.c. of each of the solutions A and B are placed in a white porcelain basin and 40 c.c. of water added; the mixture is then boiled. The sugar solution is placed in a burette and is run into the hot copper solution about 3 c.c. at a time; after each addition the solution is boiled and the precipitate allowed to settle before the next addition is made. When the blue colour has disappeared, the amount of sugar solution used is noted.

A second titration is then carried out, and all the sugar required, less 1 c.c., to effect complete reduction, is run in at once; should this prove too small an amount of sugar, more is added drop by drop until decolorization results. The process is repeated until two readings are obtained which do not differ one from the other by more than 0·2 c.c., the one being a little too high and the other a little too low; the mean of these gives the correct result.

The chief difficulty in the titration lies in the detection of the end point; this may be ascertained by allowing the precipitate to settle, and then tilting the basin so as to view the clear liquid against the white of the dish. But if the observer's colour-sense be not very critical, an error is easily made, hence various methods have been suggested to determine accurately the end point.

1. Filter off a small quantity of the solution, acidify it with acetic acid and add a little potassium ferrocyanide; the presence of unreduced copper is indicated by the formation of a brown coloration or precipitate of copper ferrocyanide.

2. Ling's reagent consists of 1 gram of ferrous ammonium sulphate and 1·5 gram of ammonium sulphocyanide dissolved

in a mixture of 10 c.c. water and 2·5 grams of strong hydrochloric acid. The solution is decolorized immediately before use by adding a few pieces of granulated zinc. A dozen drops of the reagent are placed separately on a glazed white porcelain plate and a drop of the titration mixture is, from time to time, added to one of the drops; when no pink colour is produced, the titration is complete.

3. Harrison's indicator is made by adding a little starch paste to 100 c.c. of 10 per cent solution of potassium iodide; as this solution will not keep more than a few hours, it must be freshly prepared. One c.c. of the indicator is acidified by the addition of 10 drops of acetic acid and a little of the titration mixture is added. The presence of unreduced copper is indicated by the appearance of a red or blue colour; the absence of any colour marks the end of the reaction.

EXAMPLE.—Amount of sugar solution required to decolorize 10 c.c. of Fehling's:—

| | |
|---|---|
| 11·7 c.c. . . . . | 1st reading. |
| 11·5 c.c. . . . . | 2nd ,, |
| 11·6 c.c. . . . . | mean. |

Now since

10 c.c. Fehling's ≡ ·05 gram glucose

∴ 11·6 c.c. of the solution contained ·05 gram glucose.

∴ 100 c.c. ,, ,, $\frac{\cdot 05 \times 100}{11 \cdot 6}$ ,,

= 4·31 per cent.

### *Estimation of Galactose and Mannose.*

The procedure is exactly the same as for glucose:—

10 c.c. Fehling's ≡ ·0511 gram galactose ≡ ·4307 gram mannose.

### *Estimation of Cane Sugar.*

Cane sugar does not reduce Fehling's solution; it is therefore necessary to invert it in order to make the estimation. To do this, take a known volume of the sugar solution and add a sufficiency of strong hydrochloric acid to make it about a 10 per cent solution of the acid; heat on a water bath for about a quarter of an hour, at 70° C. Then neutralize with sodium carbonate, make up to a known volume and titrate.

The inversion of cane sugar may be represented thus :—

$$C_{12}H_{22}O_{11} + H_2O = C_6H_{12}O_6 + C_6H_{12}O_6$$

The molecular weight of cane sugar is 342, and the amount of invert sugar this will give on inversion is, from the equation, 360. In other words, 1 gram of glucose corresponds to $\frac{342}{360}$ = ·95 gram of cane sugar. The titration result must therefore be multiplied by ·95 ; otherwise stated :—

10 c.c. Fehling's ≡ ·0475 gram sucrose.

### *Estimation of Maltose.*

Three points must here be remembered: firstly, that maltose will reduce Fehling's solution; secondly, that this reduction may not be complete, and therefore the maltose must be inverted before it is titrated; thirdly, that the reducing power of maltose is not the same as glucose, 1 gram of maltose having the same reducing power as ·62 gram of glucose. From the equation representing the inversion of maltose, it may be found that 1 gram of maltose gives 1·05 gram of glucose; and, as 1 gram of maltose has the same reducing power as ·62 gram of glucose, it follows that 1 gram of maltose after inversion gives an increased reducing power, viz. :—

$$1{\cdot}05 - {\cdot}62 = {\cdot}43 \text{ gram glucose,}$$
$$\therefore {\cdot}43 \text{ gram glucose} = 1 \text{ gram maltose,}$$
$$\text{and } 1 \text{ gram glucose} = \frac{1}{{\cdot}43} \text{ gram maltose,}$$
$$= 2{\cdot}32 \text{ grams maltose.}$$

The titration result, which represents glucose, must therefore be multiplied by 2·32.

### *Estimation of Mixtures of Sugars.*

In many cases it is possible to isolate the different sugars in solution, and estimate them separately by means of Fehling's solution or by some other method, and this separation must be accomplished when their action on Fehling's solution is similar. For example, it may be desired to estimate the amount of levulose and dextrose in a solution. Add to the dilute solution some ammoniacal lead acetate; both

sugars are precipitated as lead compounds. Filter off and wash the precipitate; suspend the precipitate in water and pass through it a current of carbon dioxide. The lead compound of glucose alone is decomposed, and the glucose goes into solution. Filter off and thoroughly wash the levulose lead compound, and then suspend it in water and decompose it with sulphuretted hydrogen.

Similarly, should these two sugars be mixed with cane sugar, the latter, on the addition of ammoniacal lead acetate, remains in solution, and thus is easily separated.

Inasmuch as this method is somewhat tiresome, the following methods may be followed whenever possible:—

### GLUCOSE AND SUCROSE.

1. Take 100 c.c. of the mixture and titrate with Fehling's solution.

2. Invert 100 c.c. of the mixture by the method given, and titrate.

The first operation gives the amount of glucose = $a$.

The second operation gives the original amount of glucose together with that due to the inversion of the cane sugar = $b$.

$$\therefore (b - a) \times \cdot 95 = \text{sucrose.}$$

### GLUCOSE AND MALTOSE.

Proceed exactly as for glucose and sucrose:—

$a$ = amount of sugar before inversion.
$b$ = amount of sugar after inversion.

From the reasons already given under maltose, it follows that—

$$(b - a) \times 2 \cdot 32 = \text{maltose,}$$
$$\text{and } a - (\text{maltose} \times \cdot 62) = \text{glucose.}$$

### CANE SUGAR AND MALTOSE.

Cane sugar is inverted by citric acid, while maltose is not; this fact may be made use of in the estimation:—

1. Add to 100 c.c. of the solution 5 grams of crystallized citric acid, and heat on the water bath for about one hour. Neutralize and titrate.

Reducing power = $a$.

2. Completely invert another 100 c.c. of the solution with hydrochloric acid; neutralize and titrate.

Reducing power = $b$;
then $(b - a) \times 2{\cdot}32$ = maltose,
and $(a -$ maltose $\times {\cdot}62)$ = sucrose.

GLUCOSE, CANE SUGAR, AND MALTOSE.

1. Take 100 c.c. of the solution and titrate. The result includes the glucose together with maltose.

Reducing power = $a$.

2. Take another 100 c.c. of the solution, invert with citric acid, and then titrate. The result includes the glucose, and the invert sugar obtained from the cane sugar, together with maltose.

Reducing power = $b$.

3. Take a final 100 c.c. of the solution, and completely invert with hydrochloric acid. The result represents the whole of the sugars.

Reducing power = $c$.

Following the same reasoning as before:—

$(b - a) \times {\cdot}95$ = cane sugar,
$(c - b) \times 2{\cdot}32$ = maltose,
and $a -$ (maltose $\times {\cdot}62$) = glucose.

II. ESTIMATION BY MEANS OF PAVY'S SOLUTION.

The chief disadvantage connected with the use of Fehling's solution in the estimation of glucose is the difficulty in observing the end point of the titration owing to the red precipitate of cuprous oxide; this may be overcome by using Pavy's solution, which contains ammonia which dissolves the cuprous oxide with the formation of a colourless solution. As before, two solutions are necessary.

A. 8·316 grams of pure crystallized copper sulphate are carefully weighed and dissolved in one litre of distilled water.

B. 40·8 grams Rochelle salt.
40·8 grams caustic potash.
600 c.c. strong ammonia (·880).
Distilled water to one litre.

In making up the mixture B great accuracy is not essential.

For titration 25 c.c. of A (very accurately measured) are mixed with 25 c.c. of B. The complete reduction of 50 c.c. of Pavy's solution is effected by ·025 gram of glucose.

Pavy's solution may also be prepared from Fehling's solution as follows: 120 c.c. of Fehling's are mixed with 300 c.c. of strong ammonia (·880) and 400 c.c. of 12 per cent potash solution. The mixture is then made up with distilled water to one litre.

*Method.*—Fit a 250 c.c. flask with a well-fitting cork bored with two holes, one to contain an outlet tube and the other the nozzle of the burette. Pour into the flask 50 c.c. of Pavy's solution and 50 c.c. of distilled water; mix thoroughly and introduce a little powdered glass. Dilute the sugar solution with a 10 per cent solution of ammonia, in order that 50 c.c. shall be about equivalent to 50 c.c. of the Pavy solution. Bring the Pavy solution to the boil by means of a small flame, and run in the sugar solution 1 c.c. at a time. Having thus roughly ascertained the amount of sugar required, accurate readings are to be obtained by running in nearly all the requisite sugar at once, and then drop by drop until the end point is reached.

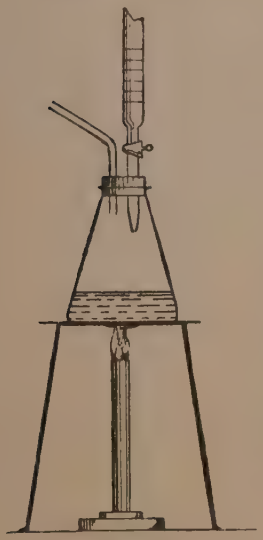


FIG. 3.

The following precautions are very important:—

1. The operation must be carried out rapidly, else all the ammonia is driven off and the cuprous oxide is precipitated.

2. The Pavy solution must be boiling throughout the titration, else air will enter the flask, owing to the lowered temperature, and the solution of cuprous oxide will be oxidized.

### III. ESTIMATION BY MEANS OF BENEDICT'S SOLUTION.

In this method the difficulty of the red precipitate of cuprous oxide obscuring the end point is overcome by carry-

ing out the reduction in the presence of potassium thiocyanate whereby the cuprous oxide is converted into an insoluble white compound, and thus the disappearance of the last trace of blue colour from the solution is easy to observe.

The solution is prepared as follows :—

200 grams sodium citrate,
200 grams crystallized sodium carbonate or 75 grams of the anhydrous salt,
125 grams potassium thiocyanate

are dissolved in water, made up roughly to 800 c,c., and filtered.

Eighteen grams of pure crystallized copper sulphate dissolved in 100 c.c. of water are poured slowly with constant stirring into the above solution. Five c.c. of a 5 per cent solution of potassium ferrocyanide are now added as a further precaution against the formation of cuprous oxide, and the whole is then carefully made up to 1000 c.c.

The above solution, which will keep indefinitely without any special precautions, is of such a strength that

25 c.c. = 0·05 gram glucose.

The titration is performed as follows :—

Twenty-five c.c. of Benedict's solution are placed in a 4 oz. flask with 3 or 4 grams of anhydrous sodium carbonate and a few lumps of broken porcelain to prevent bumping ; the mixture is kept boiling vigorously while the sugar solution is run in until the blue colour just disappears. The sugar solution may be run in rapidly at first, but towards the end it should be run in drop by drop.

The volume of solution run in contains the equivalent of 0·05 gram glucose from which the strength may be calculated.

This method is easier to work with than Fehling's solution, and gives very accurate results.

### B. *GRAVIMETRIC METHODS.*

#### *Estimation of Pentoses.*

These compounds may, according to Neuberg,* be estimated by conversion into their diphenylhydrazones,

$$C_5H_{10}O_4NN(C_6H_5)_2$$

and subsequent weighing; this method is, however, not always suitable.

The ease with which furfural can be produced from pentoses has led to the following method of estimation, which is due to Kröber †:—

A weighed quantity of substance ‡ (usually about 5 grams) is placed in a 300 c.c. flask provided with a cork bored with two holes, through one of which passes a tap-funnel, and through the other a splash preventor, such as is used in a Kjeldahl distillation. Through the tap-funnel 100 c.c. of hydrochloric acid (sp. gr. 1·06, containing about 12 per cent HCl) are then added, and the contents of the flask are distilled briskly; when 30 c.c. have passed over, the distillation is interrupted and the contents of the receiver are poured into a beaker with a 400 c.c. graduation mark; a fresh quantity of 30 c.c. of hydrochloric acid (sp. gr. 1·06) is now added through the tap-funnel, and the distillation is continued until 30 c.c. more have distilled over; the new distillate is again transferred to the beaker, 30 c.c. more acid are added to the flask, and the whole process is repeated; altogether about a dozen distillations, each lasting ten minutes, are required to carry over the last traces of furfural. In order to ascertain whether the distillate still contains furfural, a drop of the liquid is placed on a

* Neuberg: "Ber. deut. chem. Gesells.," 1900, **35**, 2243; see also Maurenbrecher and Tollens: "Ber. deut. chem. Gesells.," 1906, **39**, 3578.

† Kröber: "J. Landw.," 1900, **48**, 357, and 1901, **49**, 7. Tollens, "Z. physiol. Chem.," 1902, **36**, 239.

‡ The amount chosen should be sufficient to produce from ·03 to ·03 gram of phloroglucide.

filter paper next to a drop of aniline acetate solution ;* if no red colour appears when the two liquids come in contact with each other, the solution is free from furfural, and the distillation can be discontinued.

The furfural contained in the united distillates is then precipitated from solution by means of phloroglucinol which reacts according to the equation :—

$$\underset{90}{C_5H_4O_2} + \underset{126}{C_6H_6O_3} = C_{11}H_6O_3 + 2H_2O$$

To this end about the amount of phloroglucinol † likely to be required by the furfural obtained is dissolved in hydrochloric acid (sp. gr. 1·06), and added to the furfural solution, and the total volume is then made up to 400 c.c. with more of the same acid. The solution at once turns yellow, then becomes turbid, and, on the next day, the greenish-black precipitate of the phloroglucide is filtered off on to a tared Gooch crucible ; the precipitate is washed with 150 c.c. of water, dried for four hours at 97°, then cooled in a desiccator and weighed in a weighing bottle.‡ From the weight ($a$) of the precipitate, which under ordinary conditions should lie between 0·03 and 0·3 gram, the weight of furfural, pentose or pentosane may be calculated by substituting the value of ($a$) in one of the following formulæ :—

$a$ lies between 0·03 and 0·3 gram.

Furfural $= (a + \cdot0052) \times \cdot5185$

Pentose $= (a + \cdot0052) \times 1\cdot0075$

Pentosane $= (a + \cdot0052) \times \cdot8866$

in which ·0052 is the weight of phloroglucide, which remains in solution under the conditions of the experiment as given above.

If the precipitate weighs less than 0·03 gram or more than 0·3 gram, one of the following formulæ must be employed :—

* This is best prepared, according to Tollens, by shaking up equal volumes of aniline and water in a test tube and adding glacial acetic acid drop by drop until the turbid solution suddenly becomes clear.

† The phloroglucinol employed must be pure. To ascertain this, test as follows: Dissolve a small quantity in a few drops of acetic anhydride, heat almost to boiling and add a few drops of concentrated sulphuric acid ; a violet colour indicates the presence of diresorcinol ; if more than a faint coloration appears, the sample should be rejected.

‡ This is necessary to prevent the phloroglucide, which is hygroscopic, from absorbing moisture.

| Weight of precipitate < ·03 gram. | Weight of precipitate > 0·3 gram. |
|---|---|
| Furfural = $(a + 0{\cdot}0052) \times 0{\cdot}517$ | Furfural = $(a + 0{\cdot}0052) \times 0{\cdot}518$ |
| Pentose = $(a + 0{\cdot}0052) \times 1{\cdot}017$ | Pentose = $(a + 0{\cdot}0052) \times 1{\cdot}0026$ |
| Pentosane = $(a + 0{\cdot}0052) \times 0{\cdot}8949$ | Pentosane = $(a + 0{\cdot}0052) \times 0{\cdot}8824$ |

According to Böddener and Tollens * a considerable saving in time may be effected by precipitating the phloroglucide in hot solution, i.e. between 80 and 85°. The reaction then takes place according to the equation—

$$C_5H_4O_2 + C_8H_6O_3 = C_{11}H_4O_2 + 3H_2O$$

so that the precipitate actually weighs less than the one produced in the cold; the precipitation is, however, complete in from one and a half to two hours. The weight of furfural corresponding to the precipitate so obtained may be calculated by adding ·001 (to allow for the phloroglucide remaining in solution) and multiplying the resulting figure by 0·571. The number so obtained if multiplied by 1·935 gives the corresponding amount of pentose or if multiplied by 1·703 gives the amount of pentosane. The method is, however, not suitable if it is desired to estimate the methyl-pentosanes as distinct from the pentosanes, in which case Kröber's method as modified by Ellett † and Mayer ‡ should be employed.

### *Estimation of Glucose.*

Several methods § have been devised for employing Fehling's solution for the gravimetric estimation of sugars, each method only giving accurate results if careful attention is given to all details of manipulation.

According to Allihn two solutions are required:—

A. Prepared by dissolving 34·6 grams of pure crystallized copper sulphate in water and making up the volume to 500 c.c.

B. Containing 173 grams of Rochelle salt and 125 grams of potassium hydroxide dissolved in water and made up to 500 c.c.

Thirty c.c. of each of the two solutions A and B are mixed together in a 300 c.c. beaker, diluted with 60 c.c. of water and

* Böddener and Tollens: "J. Landw.," 1910, **58**, 232.
† Ellett: "J. Landw.," 1905, **53**, 13.
‡ Mayer: "J. Landw.," 1907, **55**, 261.
§ Soxhlet: "J. f. prakt. Chem.," 1880, [2], **21**, 227; Allihn: *id.*, 1880, [2], **22**, 63; Pflüger: "Pflüger's Archiv," 1898, **69**, 399.

heated to boiling; 25 c.c. of the sugar solution, so prepared as not to contain more than 0·25 gram of glucose, are then added, and the boiling is continued for exactly two minutes;* at the end of this time the supernatant liquid should be still blue; if not, the sugar solution was too strong and a fresh experiment must be started using a more dilute sugar solution. The liquid is then filtered through a weighed asbestos Gooch crucible which has been previously washed first with water and then successively with 10 c.c. of alcohol and a like quantity of ether, and has been dried for half an hour in a steam oven before weighing. The precipitate is then similarly washed with hot water and finally with 10 c.c. of alcohol and 10 c.c. of ether, and is dried for half an hour in a steam oven. After cooling in a desiccator, the Gooch crucible is weighed again.

The weight of cuprous oxide multiplied by the factor 0·8883 gives the weight of copper, from which the amount of dextrose may be calculated by reference to the table on pp. 102-103.

An alternative method consists in mixing as before 30 c.c. of each of the solutions A and B, diluting them with 60 c.c. of water and heating by immersing the mixture in a boiling water bath for six minutes; 25 c.c. of the sugar solution, containing not more than 0·25 gram of glucose, are then boiled and added to the copper solution; the mixture is then heated in the boiling water bath for another ten minutes. The precipitated cuprous oxide is thereupon filtered, as before, on a tared asbestos Gooch crucible, washed, dried, and weighed.

As the results obtained by different workers are liable to vary somewhat, it is best for each worker to determine for himself what results he obtains when using a glucose solution of known strength; by dividing the weight of glucose known to be in the solution by the weight of cuprous oxide obtained, a factor is found which on subsequent occasions can be used for multiplying into the weight of cuprous oxide obtained, in order to give the corresponding amount of glucose.

A sufficiently accurate result can, however, usually be obtained by employing the factors given in the following table for

* According to Pflüger this is not sufficiently long.

converting a given weight of cuprous oxide into glucose, cane sugar (after inversion), lactose, or maltose :—

| | Glucose, Levulose, or Galactose. | Cane sugar. | Lactose. | Maltose. |
|---|---|---|---|---|
| Cuprous oxide . . | ·5042 | ·4790* | ·6843 | ·8132 |
| Cupric oxide . . | ·4535 | ·4308 | ·6153 | ·7314 |
| Copper . . . | ·5634 | ·5395 | ·7709 | ·9089 |

Supposing the weight of cuprous oxide obtained by the oxidation of a lactose solution to be ·185, then the weight of lactose corresponding to this would be—

$$\cdot 185 \times \cdot 6843 = \cdot 126 \text{ gram.}$$

The chief source of error in this method lies in the possibility of the cuprous oxide containing impurities, such as silica or alumina, derived from the alkali, in which case, of course, its weight would be too high ; to overcome this objection several methods have been devised, such as reducing the cuprous oxide to metallic copper, or depositing the copper electrolytically and weighing this; or else oxidizing the cuprous oxide to cupric oxide and weighing again.

The factors for converting metallic copper and cupric oxide into sugars are also given in the above table.

### *Estimation of Glucose as Osazone.*

The following method of estimating glucose as osazone in the products of the action of malt upon starch is recommended by Davis and Ling: * Twenty c.c. of solution containing 2-3 grams of starch products per 100 c.c. are mixed with 1 c.c. of phenylhydrazine and 1·5 c.c. of 50 per cent acetic acid. After heating for an hour † over a water bath, the liquid, which has by this time evaporated to a small bulk, is filtered through a tared Gooch crucible, and the crystalline osazone is washed with 20-30 c.c. of boiling water, so that the total filtrate does not exceed 50 c.c.; the precipitate is then dried in a steam oven and weighed; under these conditions, 0·1 gram of glucose gives 0·0505 gram of glucosazone.

* Davis and Ling: "Journ. Chem. Soc., Lond.," 1904, 85, 24.

† The heating should not be continued for more than one hour.

| Copper. Mgram. | Grape Sugar. Mgram. | Copper. Mgram. | Grape Sugar. Mgram. | Copper. Mgram. | Grape Sugar. Mgram. | Copper. Mgram. | Grape Sugar. Mgram. |
|---|---|---|---|---|---|---|---|
| 10 | 6·1 | 68 | 34·8 | 126 | 64·2 | 184 | 94·2 |
| 11 | 6·6 | 69 | 35·3 | 127 | 64·7 | 185 | 94·7 |
| 12 | 7·1 | 70 | 35·8 | 128 | 65·2 | 186 | 95·2 |
| 13 | 7·6 | 71 | 36·3 | 129 | 65·7 | 187 | 95·7 |
| 14 | 8·1 | 72 | 36·8 | 130 | 66·2 | 188 | 96·3 |
| 15 | 8·6 | 73 | 37·3 | 131 | 66·7 | 189 | 96·8 |
| 16 | 9·0 | 74 | 37·8 | 132 | 67·2 | 190 | 97·3 |
| 17 | 9·5 | 75 | 38·3 | 133 | 67·7 | 191 | 97·8 |
| 18 | 10·0 | 76 | 38·8 | 134 | 68·2 | 192 | 98·4 |
| 19 | 10·5 | 77 | 39·3 | 135 | 68·8 | 193 | 98·9 |
| 20 | 11·0 | 78 | 39·8 | 136 | 69·3 | 194 | 99·4 |
| 21 | 11·5 | 79 | 40·3 | 137 | 69·8 | 195 | 100·0 |
| 22 | 12·0 | 80 | 40·8 | 138 | 70·3 | 196 | 100·5 |
| 23 | 12·5 | 81 | 41·3 | 139 | 70·8 | 197 | 101·0 |
| 24 | 13·0 | 82 | 41·8 | 140 | 71·3 | 198 | 101·5 |
| 25 | 13·5 | 83 | 42·3 | 141 | 71·8 | 199 | 102·0 |
| 26 | 14·0 | 84 | 42·8 | 142 | 72·3 | 200 | 102·6 |
| 27 | 14·5 | 85 | 43·4 | 143 | 72·9 | 201 | 103·1 |
| 28 | 15·0 | 86 | 43·9 | 144 | 73·4 | 202 | 103·7 |
| 29 | 15·5 | 87 | 44·4 | 145 | 73·9 | 203 | 104·2 |
| 30 | 16·0 | 88 | 44·9 | 146 | 74·4 | 204 | 104·7 |
| 31 | 16·5 | 89 | 45·4 | 147 | 74·9 | 205 | 105·3 |
| 32 | 17·0 | 90 | 45·9 | 148 | 75·5 | 206 | 105·8 |
| 33 | 17·5 | 91 | 46·4 | 149 | 76·0 | 207 | 106·3 |
| 34 | 18·0 | 92 | 46·9 | 150 | 76·5 | 208 | 106·8 |
| 35 | 18·5 | 93 | 47·4 | 151 | 77·0 | 209 | 107·4 |
| 36 | 18·9 | 94 | 47·9 | 152 | 77·5 | 210 | 107·9 |
| 37 | 19·4 | 95 | 48·4 | 153 | 78·1 | 211 | 108·4 |
| 38 | 19·9 | 96 | 48·9 | 154 | 78·6 | 212 | 109·0 |
| 39 | 20·4 | 97 | 49·4 | 155 | 79·1 | 213 | 109·5 |
| 40 | 20·9 | 98 | 49·9 | 156 | 79·6 | 214 | 110·0 |
| 41 | 21·4 | 99 | 50·4 | 157 | 80·1 | 215 | 110·6 |
| 42 | 21·9 | 100 | 50·9 | 158 | 80·7 | 216 | 111·1 |
| 43 | 22·4 | 101 | 51·4 | 159 | 81·2 | 217 | 111·6 |
| 44 | 22·9 | 102 | 51·9 | 160 | 81·7 | 218 | 112·1 |
| 45 | 23·4 | 103 | 52·4 | 161 | 82·2 | 219 | 112·7 |
| 46 | 23·9 | 104 | 52·9 | 162 | 82·7 | 220 | 113·2 |
| 47 | 24·4 | 105 | 53·5 | 163 | 83·3 | 221 | 113·7 |
| 48 | 24·9 | 106 | 54·0 | 164 | 83·8 | 222 | 114·3 |
| 49 | 25·4 | 107 | 54·5 | 165 | 84·3 | 223 | 114·8 |
| 50 | 25·9 | 108 | 55·0 | 166 | 84·8 | 224 | 115·3 |
| 51 | 26·4 | 109 | 55·5 | 167 | 85·3 | 225 | 115·9 |
| 52 | 26·9 | 110 | 56·0 | 168 | 85·9 | 226 | 116·4 |
| 53 | 27·4 | 111 | 56·5 | 169 | 86·4 | 227 | 116·9 |
| 54 | 27·9 | 112 | 57·0 | 170 | 86·9 | 228 | 117·4 |
| 55 | 28·4 | 113 | 57·5 | 171 | 87·4 | 229 | 118·0 |
| 56 | 28·8 | 114 | 58·0 | 172 | 87·9 | 230 | 118·5 |
| 57 | 29·3 | 115 | 58·6 | 173 | 88·5 | 231 | 119·0 |
| 58 | 29·8 | 116 | 59·1 | 174 | 89·0 | 232 | 119·6 |
| 59 | 30·3 | 117 | 59·6 | 175 | 89·5 | 233 | 120·1 |
| 60 | 30·8 | 118 | 60·1 | 176 | 90·0 | 234 | 120·7 |
| 61 | 31·3 | 119 | 60·6 | 177 | 90·5 | 235 | 121·2 |
| 62 | 31·8 | 120 | 61·1 | 178 | 91·1 | 236 | 121·7 |
| 63 | 32·3 | 121 | 61·6 | 179 | 91·6 | 237 | 122·3 |
| 64 | 32·8 | 122 | 62·1 | 180 | 92·1 | 238 | 122·8 |
| 65 | 33·3 | 123 | 62·6 | 181 | 92·6 | 239 | 123·4 |
| 66 | 33·8 | 124 | 63·1 | 182 | 93·1 | 240 | 123·9 |
| 67 | 34·3 | 125 | 63·7 | 183 | 93·7 | 241 | 124·4 |

| Copper. Mgram. | Grape Sugar. Mgram. | Copper. Mgram. | Grape Sugar. Mgram. | Copper. Mgram. | Grape Sugar. Mgram. | Copper. Mgram. | Grape Sugar. Mgram. |
|---|---|---|---|---|---|---|---|
| 242 | 125·0 | 298 | 155·4 | 354 | 186·6 | 410 | 218·7 |
| 243 | 125·5 | 299 | 156·0 | 355 | 187·2 | 411 | 219·3 |
| 244 | 126·0 | 300 | 156·5 | 356 | 187·7 | 412 | 219·9 |
| 245 | 126·6 | 301 | 157·1 | 357 | 188·3 | 413 | 220·4 |
| 246 | 127·1 | 302 | 157·6 | 358 | 188·9 | 414 | 221·0 |
| 247 | 127·6 | 303 | 158·2 | 359 | 189·4 | 415 | 221·6 |
| 248 | 128·1 | 304 | 158·7 | 360 | 190·0 | 416 | 222·2 |
| 249 | 128·7 | 305 | 159·3 | 361 | 190·6 | 417 | 222·8 |
| 250 | 129·2 | 306 | 159·8 | 362 | 191·1 | 418 | 223·3 |
| 251 | 129·7 | 307 | 160·4 | 363 | 191·7 | 419 | 223·9 |
| 252 | 130·3 | 308 | 160·9 | 364 | 192·3 | 420 | 224·5 |
| 253 | 130·8 | 309 | 161·5 | 365 | 192·9 | 421 | 225·1 |
| 254 | 131·4 | 310 | 162·0 | 366 | 193·4 | 422 | 225·7 |
| 255 | 131·9 | 311 | 162·6 | 367 | 194·0 | 423 | 226·3 |
| 256 | 132·4 | 312 | 163·1 | 368 | 194·6 | 424 | 226·9 |
| 257 | 133·0 | 313 | 163·7 | 369 | 195·1 | 425 | 227·5 |
| 258 | 133·5 | 314 | 164·2 | 370 | 195·7 | 426 | 228·0 |
| 259 | 134·1 | 315 | 164·8 | 371 | 196·3 | 427 | 228·6 |
| 260 | 134·6 | 316 | 165·3 | 372 | 196·8 | 428 | 229·2 |
| 261 | 135·1 | 317 | 165·9 | 373 | 197·4 | 429 | 229·8 |
| 262 | 135·7 | 318 | 166·4 | 374 | 198·0 | 430 | 230·4 |
| 263 | 136·2 | 319 | 167·0 | 375 | 198·6 | 431 | 231·0 |
| 264 | 136·8 | 320 | 167·5 | 376 | 199·1 | 432 | 231·6 |
| 265 | 137·3 | 321 | 168·1 | 377 | 199·7 | 433 | 232·2 |
| 266 | 137·8 | 322 | 168·6 | 378 | 200·3 | 434 | 232·8 |
| 267 | 138·4 | 323 | 169·2 | 379 | 200·8 | 435 | 233·4 |
| 268 | 138·9 | 324 | 169·7 | 380 | 201·4 | 436 | 233·9 |
| 269 | 139·5 | 325 | 170·3 | 381 | 202·0 | 437 | 234·5 |
| 270 | 140·0 | 326 | 170·9 | 382 | 202·5 | 438 | 235·1 |
| 271 | 140·6 | 327 | 171·4 | 383 | 203·1 | 439 | 235·7 |
| 272 | 141·1 | 328 | 172·0 | 384 | 203·7 | 440 | 236·3 |
| 273 | 141·7 | 329 | 172·5 | 385 | 204·3 | 441 | 236·9 |
| 274 | 142·2 | 330 | 173·1 | 386 | 204·8 | 442 | 237·5 |
| 275 | 142·8 | 331 | 173·7 | 387 | 205·4 | 443 | 238·1 |
| 276 | 143·3 | 332 | 174·2 | 388 | 206·0 | 444 | 238·7 |
| 277 | 143·9 | 333 | 174·8 | 389 | 206·5 | 445 | 239·3 |
| 278 | 144·4 | 334 | 175·3 | 390 | 207·1 | 446 | 239·8 |
| 279 | 145·0 | 335 | 175·9 | 391 | 207·7 | 447 | 240·4 |
| 280 | 145·5 | 336 | 176·5 | 392 | 208·3 | 448 | 241·0 |
| 281 | 146·1 | 337 | 177·0 | 393 | 208·8 | 449 | 241·6 |
| 282 | 146·6 | 338 | 177·6 | 394 | 209·4 | 450 | 242·2 |
| 283 | 147·2 | 339 | 178·1 | 395 | 210·0 | 451 | 242·8 |
| 284 | 147·7 | 340 | 178·7 | 396 | 210·6 | 452 | 243·4 |
| 285 | 148·3 | 341 | 179·3 | 397 | 211·2 | 453 | 244·0 |
| 286 | 148·8 | 342 | 179·8 | 398 | 211·7 | 454 | 244·6 |
| 287 | 149·4 | 343 | 180·4 | 399 | 212·3 | 455 | 245·2 |
| 288 | 149·9 | 344 | 180·9 | 400 | 212·9 | 456 | 245·7 |
| 289 | 150·5 | 345 | 181·5 | 401 | 213·5 | 457 | 246·3 |
| 290 | 151·0 | 346 | 182·1 | 402 | 214·1 | 458 | 246·9 |
| 291 | 151·6 | 347 | 182·6 | 403 | 214·6 | 459 | 247·5 |
| 292 | 152·1 | 348 | 183·2 | 404 | 215·2 | 460 | 248·1 |
| 293 | 152·7 | 349 | 183·7 | 405 | 215·8 | 461 | 248·7 |
| 294 | 153·2 | 350 | 184·3 | 406 | 216·4 | 462 | 249·3 |
| 295 | 153·8 | 351 | 184·9 | 407 | 217·0 | 463 | 249·9 |
| 296 | 154·3 | 352 | 185·4 | 408 | 217·5 | | |
| 297 | 154·9 | 353 | 186·0 | 409 | 218·1 | | |

### C. *POLARIMETRIC METHODS.*

The presence of an asymmetric carbon atom confers upon a compound the property of optical activity, by which is meant the power of the substance to rotate to the right or to the left the plane of a beam of circularly polarized light passing through it.

The polarimeter is much used in ascertaining the strength of sugar solutions, but before describing the mode of using it, it is desirable to consider briefly the principles which are involved.

When a ray of light enters a crystal of any system other than the cubical, it is broken up into two rays, the ordinary and the extra-ordinary, provided the beam of light is not coincident with the optical axis of the crystal. This phenomenon is known as double refraction.

These two rays, the ordinary and the extra-ordinary, do not behave similarly; the former conforms to the ordinary laws of refraction, but the latter does not; further, the two rays are polarized in directions at right angles to one another.

In order to make use of these facts, it is necessary to be able to examine the extra-ordinary ray alone; that is, the two rays must be separated one from the other. This is effected by a Nicol's prism, which consists of two plates of Iceland spar fixed together by means of Canada balsam. A ray of light enters one side of the prism, and is broken up into the ordinary and the extra-ordinary ray; on reaching the layer of balsam, the former is totally reflected, whilst the latter passes on through the other plate and emerges at the side opposite to its entry. If a second Nicol be placed in the path of this ray, the latter will pass through in different amounts according to the angle which the second prism makes with the first. If the interposed Nicol be parallel to the first Nicol, the ray will pass through entirely; if the second Nicol be rotated, the light passing through will be less and less in amount until, when the two prisms are at right angles to each other, no light passes at all. If the rotation be continued, the light will again pass through in gradually increasing quantities until the prism has been rotated through an angle of 180°

from its original position, when the whole light will again pass through freely.

Many liquids and solutions of solids possess what is known as optical activity, which means that they can rotate the plane of vibration of a ray of polarized light passing through them; so that, on emergence from the liquid, the new plane is inclined either to the right or to the left of the original plane.

This is known as the rotation of the plane of polarized light.

*Laurent's Half-Shadow Polarimeter.*—This apparatus consists of a tube containing two Nicol's prisms, of which one is fixed and is known as the polarizer, while the other can be rotated and is called the analyser. A quartz plate which covers half the field of vision is fixed just behind the polarizer.

The liquid or solution to be examined is contained within a glass tube with polished ends, and is placed in position between the quartz plate and the analyser. The analyser is fixed in a tube which can be rotated, the degree of rotation being read from a divided circle. Leaving out of consideration the quartz plate, the beam of polarized light passes through the liquid and so becomes rotated; it follows, therefore, that the vibration plane of the analyser will no longer be at right angles to the plane of polarization of the light striking it, therefore light will enter the analyser, and in order to bring about complete extinction, the analyser must be rotated either to the right or to the left. This angle of rotation is a measure of the optical activity of the substance under observation, and according to the direction of rotation, the substance is termed dextro- or lævo-rotatory. In Laurent's polarimeter the illumination is obtained from a sodium flame, and this light before entering the tube containing the liquid must pass through the plate of quartz. When the instrument is set in the zero position, the whole field is equally illuminated, but on introducing the liquid, one half of the field becomes the darker; equal illumination can be obtained by rotating the analyser. If this position be passed, the field is once more unequally illuminated, but in a reverse manner, that is to say, the half which was originally dark is now light, and vice versa.

As the exact position of equal illumination is somewhat difficult to determine, several readings should be made and the mean of these taken as the correct value.

The *specific rotation* of a substance is defined as the angular rotation produced by a column of liquid 1 dm. in length, which contains 1 gram of the active substance in each cubic centimetre. This quantity is expressed by the symbol $a^{20}_{D}$, the numeral indicating the temperature at which the measurements were made, and the letter D standing for the yellow line of the sodium flame which is used as the source of illumination. The use of this quantity $a_D$ for determining the number of grams of active substance in a given solution will be rendered apparent from the following considerations.

Supposing we have a solution containing an unknown number of grams, $m$, of active substance per c.c., and we fill a tube of length $l$ dm.* with this solution and observe its angular rotation to be $a$.

If a layer 1 dm. long containing 1 gram { of substance in 1 c.c. of liquid produces a rotation } $a_D$

Then " " $l$ " " " 1 " " " " " $la_D$

∴ " " $l$ " " " $m$ " " " " $mla_D$

And this would be the observed angle of rotation ($a$)

$$\therefore a = m \times l \times a_D$$

$$\therefore m = \frac{a}{l \times a_D}$$

The angle of rotation is determined as follows:—

1. Find the zero reading when no solution is between the polarizer and analyser. For this purpose the mean of at least three readings, differing by only two or three minutes, should be taken.

2. Fill the tube with the liquid, taking care to avoid the introduction of air-bubbles.

3. Insert the tube and determine the new reading at which the illumination of both halves of the field is equal. The mean of three readings should again be taken.

The difference between the initial and the final readings is the angle of rotation.

The following experiment performed on a solution known to contain glucose may be quoted in illustration of the method:—

* The length of the tube must be expressed in *decimetres*.

Initial reading of polariscope, without any solution = 0° 30′
Final " " " with glucose " = 3° 45′
Difference ($\alpha$) = 3° 15′ or 3·25°
Length of tube containing the solution ($l$) = 2 dms.
Specific rotation of glucose ($\alpha^{20}_{D}$) = 52·5°

$$\text{From which } m = \frac{3{\cdot}25}{2 \times 52{\cdot}5} = {\cdot}0309$$

∴ the strength of the solution is 3·09 per cent.

It is of course obvious that correct values can only be obtained by this method on the assumption that the liquid contains only a single optically active substance.

Some substances, e.g. glucose, exhibit the phenomenon of muta-rotation, that is to say, the rotation of their solutions varies according to the length of time that they have been made up; the maximum rotation is given by a freshly-made solution, but the rotatory power gradually decreases until it finally becomes steady. The attainment of the final condition is greatly accelerated by warming the solution in the presence of a little alkali, but the solution must of course be cooled before a reading is taken.

### FURTHER REFERENCES.

Armstrong: "The Simpler Carbohydrates and Glucosides," London, 1919; Mackenzie: "The Sugars and their Simpler Derivatives," London, 1913.

## POLYSACCHARIDES.

The second great group of carbohydrates, namely the non-sugars or polysaccharides, are substances of high molecular weight, mostly amorphous and insoluble in water. Like the di- and tri-saccharides, the polysaccharides on hydrolysis break up into sugars containing five or six carbon atoms, and they may therefore be looked upon as anhydrides of these substances.

In the absence of any exact knowledge regarding their molecular weights, their formulæ are written $(C_6H_{10}O_5)_n$ or $(C_5H_8O_4)_n$ according as they give rise to hexoses or pentoses on hydrolysis. The value of "n" has not been determined as yet for any particular case, but there is reason to believe that it is fairly high. The various methods adopted for the elucidation of this point have led to such widely different results

that a description of them here would not serve any useful purpose.

### CLASSIFICATION.

The polysaccharides may be classified as follows:—

I. Starches and Dextrins, including Glycogen, Inulin, Mannane and Galactane $(C_6H_{10}O_5)_n$.

II. Gums, which comprise (*a*) Natural Gums and Pentosanes; (*b*) Mucilages and Pectic Bodies.

III. Celluloses $(C_6H_{10}O_5)_n$.

## STARCHES.

The general formula for all substances belonging to this group is $(C_6H_{10}O_5)_n$, which indicates that on hydrolysis they yield hexoses; for this reason they may be termed hexosanes. The hexoses produced however are different, and the group may therefore be subdivided as under, the basis of the classification being the nature of the sugar.

| | |
|---|---|
| Starches or Hexosanes. | Dextrosanes.—Ordinary starch, Dextrin, Glycogen, Paradextrane, etc. |
| | Levulosanes.—Inulin, Phlein, Graminin, Triticin, etc. |
| | Mannosanes.—Mannane, Paramannane, Mannocellulose. |
| | Galactosanes.—Galactane, Paragalactane. |

## DEXTROSANES.

### *Starch or Amylum.*

Starch is one of the most widely distributed substances in the vegetable kingdom; it may be found in green leaves as a temporary reserve of the photosynthetic products; as a more or less permanent reserve food-material it occurs in seeds and fruits, where it is not infrequently accompanied by other reserves, for instance proteins; in the vegetative parts, such as tubers, the living cells of the pith, medullary rays, and cortex of roots and stems; and also in the latex of certain plants, e.g. *Euphorbia*. When especially stored, the amount of starch may be considerable; thus in cereals it may form from 50 to 70 per cent of the dry weight of the grains, and in potatoes from 15 to 30 per cent of the dry weight of the tubers. As is well known, starch grains from different sources show much variety in size and shape, and occur in

association with plastids, in which, as Schimper demonstrated, they have their origin. Not only are the microscopic characters of starch grains of diagnostic value, but the different varieties of starch can be grouped into generic, specific, and varietal classes which correspond with the classification of plants based on the ordinary morphological features.*

Many monocotyledonous plants are characterized by the absence of starch, for example *Scilla nutans*, *Phleum pratense*, *Allium*, etc., but in some of these cases starch granules may occur in the guard-cells of the stomates, in the bundle sheath of the leaves, and also in the bulbs at the base of the growing shoots; further, in certain plants which normally form sugar, e.g. *Musa*, *Hemerocallis*, and *Muscari*, starch will appear when much sugar accumulates. On the other hand, many members of this same class of plants are fairly constant starch producers, e.g. *Lilium tigrinum*, *Pontederia cordata*, *Ananas sativa*, *Canna indica*, *Tradescantia virginica*, *Juncus communis*, and *Alisma Plantago*. There are many peculiarities in this occurrence of starch in the Monocotyledons; for instance, in *Scilla nutans* it is absent, whilst in *Scilla siberica* it is quite abundant; further, the former plant, if fed with cane sugar in a solution of suitable strength, does not form it, while, on the other hand, starch-free plants of *Scilla siberica* under the same treatment do form starch, the experiment being carried out in the absence of light. In the Mycetozoa, in which starch is normally absent, starch formation may be induced under the influence of acid and a supply of sugars.† In the plant starch occurs, as is well known, in the form of variously shaped microscopic bodies composed of concentric layers; the granules have an organized structure and possess the power of double refraction.

### *Preparation of Starch.*

The method of preparation varies according to the source employed. From wheat flour it may be obtained by stirring up this material thoroughly with water, and allowing the mix-

* Reichert: "Amer. Journ. Bot," 1916, 3, 91.
† Boas: "Biochem. Zeitsch.," 1917, 78, 308.

ture to stand until the gluten contained in the flour undergoes fermentation, when it dissolves and may be removed by washing. On a small scale the separation is most conveniently effected by kneading some flour in a muslin bag which is held under a stream of water. The starch granules are hereby washed through the muslin, while the gluten remains behind in the bag as a sticky grey mass.

Starch may also be obtained from potatoes by macerating them with water and separating the non-starchy material from the starch by filtration. The starch is then allowed to settle at the bottom of the water, when it is collected and dried.

### *Purification.*

Malfitano and Moschkoff * give the following method for the purification of starch: A one per cent colloidal solution of starch is frozen and then allowed to melt. When melted, most of the starch is deposited in a floccular precipitate, whilst the clear liquid contains some starch and the greater part of the mineral impurities. On repeating the operation four or five times, the purified product yields less than ·02 per cent of ash.

### *Properties.*

Air-dried starch contains a considerable quantity of water, as much as 20 per cent being not uncommon; it can be made to part with this water by carefully heating to 100°. If heated to about 200° it is converted into a sticky soluble substance, which is probably a mixture of isomeric substances of the empirical formula $C_6H_{10}O_5$, known as British gum or dextrin (q.v.).

Starch is quite insoluble in cold water, but if heated with water the granules swell and burst, a slimy opalescent mass known as starch paste being formed. The consistency of this paste varies of course with the concentration, and also with the particular kind of starch employed; this may be accounted for by assuming that some starches are richer than others in the constituent which produces the viscosity (p. 115). If a dilute starch paste be filtered, a gelatinous residue remains on the filter paper; the filtrate contains some starch, since it gives a

* Malfitano and Moschkoff: "Compt. rend.," 1910, 151, 817.

blue colour with iodine, but it is doubtful whether the liquid is a true solution; it is more likely a colloidal solution in which the particles are sufficiently small to pass through the pores of the filter paper.

With regard to the chemical nature of starch granules there are considerable differences of opinion. The researches of Nägeli have shown that when starch is treated with dilute hydrochloric acid, malt extract, or saliva, a considerable portion goes into solution, leaving a transparent skeleton undissolved. The soluble portion, which gives a blue colour with iodine, Nägeli regarded as the true starch constituent of the granule, and named it granulose; on the other hand, the undissolved skeleton, which is not turned blue by iodine, he considered to be of a cellulose nature, and called it starch cellulose or amylo-cellulose.

On the other hand, Meyer * was of opinion that starch granules consisted essentially of two substances known respectively as $\alpha$ and $\beta$ amylose. The former, which was insoluble, he regarded as an anhydride which could be converted into the soluble $\beta$ variety by the action of superheated steam.

He also thought that when starch is acted upon by hydrochloric acid it is converted into amylo-dextrin, and considered that amylo-cellulose, which Nägeli regarded as an original constituent of the starch granule, was in reality identical with amylo-dextrin, and therefore a secondary product of the action of acid on the amylose.

According to the view of recent workers, notably Maquenne and Roux,† and Fernbach and Wolff,‡ starch granules consist of two substances: amylo-cellulose, or amylose, as they describe it later, and amylo-pectin. The term amylo-cellulose is, however, employed in a different sense from that assigned to it by Nägeli. It is, according to these authors, the principal constituent, and is partly soluble in boiling water and completely soluble in water under pressure; in solution it gives a blue colour with iodine, and is converted into maltose by malt, but in the solid state it is not acted upon by these reagents. The soluble form is produced by heating the insoluble one with

* Meyer: "Unters ü. d. Stärkekörner," Jena, 1895.
† Maquenne and Roux: "Compt. rend.," 1903, 137, 88; 1905, 140, 1303.
‡ Fernbach and Wolf: *id.*, 1904, 138, 819.

water under pressure, and the insoluble form may be recovered from the solution by cooling, a process which is known as "reversion". The insoluble amylo-cellulose is probably identical with the substance described by Nägeli under that name, in that it is not coloured by iodine; it is, however, not regarded as differing essentially from the soluble form (Nägeli's granulose), but rather as being a polymer of it or a different crystalline variety.

The second constituent, amylo-pectin, is a mucilaginous substance of an entirely different nature, which is not coloured blue by iodine and dissolves in malt extract, without, however, being converted into a sugar; it swells up without dissolving when heated with water. According to Maquenne* and Roux, it is amylo-pectin which produces the gelatinization of starch in the form of starch paste, which substance may therefore be regarded as a colloidal solution of amylo-cellulose (amylose) thickened by an insoluble gelatinized slimy material, the amylo-pectin. Amylo-pectin, moreover, tends to retard the reversion of soluble amylo-cellulose into the insoluble form, and hence there is a quantity of the soluble form present in the starch granule which is able to dissolve in boiling water; when, however, the amylo-pectin is removed, the pure insoluble amylo-cellulose or amylose (as the authors prefer to call it) is produced.†

A new form of soluble starch has recently been described by Fernbach.‡ It is obtained by pouring a 1 or 2 per cent aqueous suspension of potato starch into a large excess of pure acetone and shaking vigorously; a flocculent precipitate is thus obtained, which, when filtered and ground up in a mortar with more acetone and then dried in a vacuum, yields a light white powder which is completely soluble in cold water. The aqueous solution passes through filter paper and yields a very pure blue colour with iodine.

Brief mention may be made of the ideas held regarding the physical nature of starch grains. As is well known, the gran-

* Maquenne: "Bull. Soc. Chim.," Paris, 1906, [3], **35**, 1, and "Ann. Chim. Phys.," 1904, [8], **2**, 109; Maquenne and Roux: "Ann. Chim. Phys." 1906, [8], **9**, 179.

† See also Gruzewska: "Journ. Phys. Path. Gen.," 1912, **14**, 7.

‡ Fernbach: "Compt. rend.," 1912, **155**, 617

ules not infrequently exhibit a more or less well-marked stratification which years ago was thought to correspond to the alternation of day and night.

The "apposition" theory held that new layers were added to those already formed, each layer being separated from the next by a thin film of air. Nägeli came to the conclusion that the lamellation was due to the differences in the water-content of the several layers, and that the grain was made up of minute particles, the micellæ, which are of the prismatic order, surrounded by a film of water and embedded in a matrix. The growth of the grain took place by a process of intussusception, that is to say, new material was intercalated between the micellæ, and either gave rise to new micellæ, or was used up in increasing the size of the old ones. Schimper expressed the idea that the grains were really of a sphæro-crystalline nature, which view was modified by Meyer, who says that the grain is made up of two kinds of needle-shaped crystals composed respectively of $\alpha$ and $\beta$ amylose; he also states that in those grains which are coloured red with iodine, for example those found in the cells of the root-cap of *Allium Cepa*, in the seed-coats of *Chelidonium* and in *Oryza sativa*, var. *glutinosa*, dextrin and amylo-dextrin occur. On the other hand, the ordinary grains which are coloured blue with iodine, are made up almost entirely of sphæro-crystals of amylose arranged in layers.

According to Kraemer,* the starch grains of the potato are composed of colloid and crystalloid substances arranged in lamellæ which are distinct and separate one from the other. At the point of origin of growth, the hilum, and in the alternate lamellæ, the colloid preponderates and is associated with the crystalloid cellulose; in the other lamellæ the crystalloid granulose is in the greater proportion. He also states that the peripheral layer is elastic and porous, and may be an anhydride of cellulose. Dennison also has expressed the view that the outer layer of the grain is different from the more internal parts, and may be a carbohydrate not fully polymerized to starch.

The view that both crystalloid and colloid materials occur in the starch grain is held by many, thus Mellanby,† by the

* Kraemer: "Bot. Gaz.," 1902, 34.
† Mellanby: "Biochem. Journ.," 1919, 13, 28.

action of colloidal iron, finds that the amylo-granulose of Nägeli can be separated into three fractions, $\alpha$, $\beta$, and $\gamma$. The $\alpha$ fraction is precipitated by colloidal iron alone, the $\beta$ portion by colloidal iron in the presence of electrolytes, and the $\gamma$ fraction is not precipitated by colloidal iron under any circumstances. From the action of iodine in the presence of electrolytes he finds that starch contains an insoluble constituent (amylo-cellulose) which does not react with iodine, that all the soluble constituents are precipitated by iodine if electrolytes are present, and that the final fraction thrown down by iodine gives a brown coloration with that reagent. It is therefore concluded that starch contains a number of polymers varying in complexity from amylo-dextrin to amylo-cellulose; the main bulk of the grain is made up of amylo-granulose $\alpha$, the relative amount of dextrin and cellulose being small. It was found that the reaction of iodine with starch is quantitative, approximately 1600 gm. starch being equivalent to 127 gm. of iodine. The opinion regarding the crystalloid structure of starch is not universally held, Fischer,* who believes that the grain is composed of colloid substances, being a dissentient.

According to Harrison,† starch grains contain no starch which is really soluble in water; the differences which obtain between the inner and superficial layers are physical rather than chemical, and are due to the varying conditions under which they are formed.

### *Action of Acids on Starch.*

The action of acids on starch varies according to the strength of the acid, the duration of the action, and the temperature of the experiment. To complicate matters, there are considerable divergences in regard to the interpretation of the results obtained by the different workers. As an illustration of the very different effects which may be produced under different conditions, the following experiments may be quoted.

By acting on starch at the ordinary temperature with

* Fischer: "Beih. bot. Centr.," 1905, 181.
† Harrison: "Journ. Soc. Dyers.," 1916, 32, 40.

12 per cent commercial hydrochloric acid for twenty-four hours, Brown and Morris found that granules, while retaining their external features, had acquired the power of dissolving in hot water without the formation of paste. The addition of alcohol to such a solution caused the immediate precipitation of a white substance known as soluble starch, which is turned blue by iodine, is strongly dextro-rotatory, $[\alpha]_D = 202°$, and does not reduce Fehling's solution. On the other hand, if starch is boiled for some time with dilute hydrochloric acid, it is converted into glucose, a fact which is made use of in estimating starch.

That maltose is also produced as an intermediate product of the acid hydrolysis of starch has been shown by Fernbach and Schoen,* and also by Weber and Macpherson,† who have proved it to be present in commercial glucose (see p. 73). Accompanying the conversion of starch into glucose there is, however, the formation of varying quantities of gummy substances known as dextrins (q.v.); it is, however, not known for certain whether these dextrins are formed directly by the action of the acid on the starch, or whether they are produced by the condensing action of the acid on the glucose already formed; there is moreover great difference of opinion with regard to the nature and number of these substances which are formed.

### *Action of Malt or Diastase on Starch.*

The action of malt extract or diastase (see p. 371), like that of mineral acids, is primarily a hydrolytic one in which the starch is converted into a sugar; but the diastase does not carry the hydrolysis so far as the acid, the sugar produced being a disaccharide, maltose, and not a monosaccharide. At the same time some dextrin is also formed. Although the mechanism of this reaction is very complex and has led to a great deal of discussion, the ultimate change may be conveniently represented by the following formulæ:—

$$\underset{\text{Starch}}{(C_6H_{10}O_5)_n} + H_2O \rightarrow \underset{\text{Maltose}}{C_{12}H_{22}O_{11}} + \underset{\text{Dextrin}}{(C_6H_{10}O_5)_x}$$

* Fernbach and Schoen: "Bull. Soc. Chim.," 1912, [iv.], 11, 303.
† Weber and Macpherson: "J. Amer. Chem. Soc.," 1895, 17, 312.

*Action of Bacteria on Starch.*

By the action of *Bacillus macerans* on 5 per cent starch paste, Schardinger* has obtained two crystalline substances which he describes as $\alpha$ and $\beta$ dextrin, the former of which gives with iodine a dark green compound crystallizing in needles, whilst the latter gives dark red-brown prisms. Pringsheim and Langhans† ascribe to these compounds the formulæ $(C_6H_{10}O_5)_4$ and $(C_6H_{10}O_5)_6$ respectively; they have obtained from the former a crystalline disaccharide $(C_6H_{10}O_5)_2$, and from the latter a crystalline trisaccharide $(C_6H_{10}O_5)_3$. All these four compounds have a sweet taste and, according to the authors, they are representatives of a new class of crystalline polysaccharides which they term amyloses.‡ The substances are accordingly named di-, tri-, tetra-, and hexa-amylose. In a subsequent communication Pringsheim and Eissler§ describe two further substances, isotriamylose and isodiamylose, which are isomeric with tri- and diamylose respectively.

*Reactions.*

1. The appearance of the grains under the microscope and their action on polarized light in the presence of water are well known.

2. The most characteristic reaction of starch is the blue colour produced with iodine. The composition of this blue substance varies; it contains, on an average, about 18 per cent iodine, and cannot be formed unless a small quantity of hydriodic acid, which is always present in small amounts in ordinary solutions of iodine, be present. The blue colour is discharged on heating the solution, but reappears on cooling. The dried substance may, however, be heated to 100° without undergoing alteration. It is stated that those parts of the grain which are particularly rich in granulose are the most affected by the iodine.

If the starch grains are very small, or relatively so few in number that they might easily be overlooked, Meyer's pro-

* Schardinger: "Zeitsch. f. Natur. u. Genussm.," 1903, 6, 874.

† Pringsheim and Langhans: "Ber. deut. chem. Ges.," 1912, 45, 2533.

‡ The choice of this term is unfortunate in view of the various uses to which it has already been put by other authors, such as Meyer, Maquenne, and Roux, etc. (see pp. 115, 116).

§ Pringsheim and Eissler: "Ber. deut. chem. Gesells.," 1913, 46, 2959.

cedure may be followed. A section of the material to be examined is cut, and is first treated with a fairly dilute solution of iodine in potassium iodide, the excess of the reagent is then removed, and the section is irrigated with a concentrated aqueous solution of chloral hydrate. This causes the starch grains to swell, and at the same time the other cell contents are dissolved, as are also the starch grains in time.

3. For microscopic work, the action of dilute aqueous solutions of gentian violet and of safranin is sometimes useful, as they stain the colloidal parts more deeply.

4. Starch grains are insoluble in cold water, but in hot water they swell up and form an opalescent solution which, if strong enough, will on cooling eventually form a paste.

5. Starch is precipitated from its aqueous solution by alcohol* or by basic lead acetate (cf. Inulin and Dextrin).

6. Potash causes the grains to swell and finally to dissolve.

7. Boil a little starch paste solution with a few drops of dilute sulphuric acid in a test tube, and from time to time remove a little of the solution, cool it and test with iodine solution; when the starch has been converted into dextrin the blue colour at first formed will give way to a plum colour. If boiled too long only dextrose will remain which gives no colour with iodine. The solution will, however, after making alkaline, reduce Fehling's solution.

8. Cautiously heat a little starch on a porcelain basin until it has acquired a light fawn colour. Cool and extract with cold water, and filter; the dextrin produced being soluble in cold water is thus separated from the starch. On adding iodine to the solution a plum colour is produced.

### *Estimation of Starch.*

A colorimetric method of estimating starch, depending on the depth of the blue colour produced with iodine, has been described by Dennstedt and Voigtländer.†

The following method depending on the hydrolysis of starch by hydrochloric acid and the subsequent estimation of the glucose produced, is only reliable if there are no pentosanes

* Due, according to Harrison (*loc. cit.*), to the presence of inorganic salts.

† Dennstedt and Voigtländer: "Ber. deut. chem. Gesells.," 1895, **28**, R., 1025.

or other substances present which on hydrolysis would yield reducing sugars.

About 3 grams of the substance in as fine a state of division as possible are covered with 50 c.c. of cold water and shaken at frequent intervals; after an hour the insoluble portion is filtered off and washed with water until the total filtrate measures 250 c.c.; the addition of a little alumina shaken up with water will frequently facilitate clear filtration. The soluble carbohydrates contained in the filtrate may if desired be determined both before and after inversion.

The residue remaining on the filter paper is then transferred to a flask with a 250 c.c. graduation mark and heated for two and a half hours under a reflux condenser with 200 c.c. of water and 20 c.c. of hydrochloric acid (sp. gr. 1·125). After cooling, the solution is neutralized with caustic soda and made up to 250 c.c., whereupon it is filtered and the amount of glucose contained in an aliquot portion of the filtrate is estimated by Fehling's or Benedict's solution. The amount of glucose found when multiplied by 0·9 gives the weight of starch.

The following method for the estimation of starch in barley is due to Horace T. Brown *:—

Five grams of the powdered or crushed grain are extracted for three hours in a Soxhlet extractor with alcohol (sp. gr. 0·90); the residue is then thoroughly boiled with 100 c.c. of water, and, after cooling, to 57°, 10 c.c. of active malt extract are added and the mixture is set aside for one hour; it is thereupon boiled and filtered into a flask with a 200 c.c. graduation mark; the residue is thoroughly washed with water, and, after cooling, the filtrate and washings are made up to 200 c.c. The cupric reduction of 20 c.c. of the solution is determined under the conditions laid down by Brown, Morris and Millar,† the maltose being calculated according to Table XI in that paper (*loc. cit.*, p. 100), after correction for the reduction due to the malt extract. The starch equivalent to this maltose is then ascertained by assuming that 84·4 parts of maltose correspond to 100 parts of starch.

The malt extract is prepared by digesting 10 grams of fresh finely-ground malt for two to three hours with 200 c.c. of water and filtering.

* Horace T. Brown: "Trans. Guiness Research Lab.," 1903, **1**, 89.
† Brown, Morris and Millar: "J. Chem. Soc., Lond.," 1897, **71**, 94.

A method of starch estimation due to von Fellenberg* depends on the solution of the starch in a hot solution of calcium chloride, its precipitation by iodine and the decomposition of the iodine precipitate by alcohol.

## DEXTRINS.

The term dextrin is applied to substances which are polymeric with starch and are formed from it by the action of heat alone or of diastase or mineral acids. In the plant dextrins may occur as transitory substances whenever starch is being acted upon by diastase; further, certain dextrins may occur in a more permanent form. Thus the sap of the epidermal cells of *Arum italicum* turn reddish-violet on the application of iodine. The aqueous extract of such cells gives on evaporation a transparent sticky substance. This also gives with iodine a violet coloration; after boiling, the colour reaction with iodine is red, and after digestion with diastase a reducing sugar is found. A similar substance—termed soluble starch—has been described as occurring in the epidermis of *Saponaria officinalis* and also in Fungi. It must, however, be borne in mind that the glucoside saponarin,† $C_{21}H_{24}O_{12}$, is not uncommon in the epidermis of leaves of many plants, e.g. *Saponaria officinalis* itself, and as it gives a blue to violet coloration with iodine it is not unlikely that, in some cases, what has been described as soluble starch is really saponarin.

As already mentioned, the question of the formation of dextrins from starch by the action of diastase has been the subject of a great many researches, and has, at different times, resulted in the postulation of the existence of a large variety of dextrins and intermediate products, such as amylo-, achroo-, erythro-, and malto-dextrin, amylases, amyloins, glycoamylins, etc., many of which did not survive for long.

The chief facts observed during the action of malt extract on starch may be very briefly summarized as follows. If, say, a 10 per cent starch paste is left in contact with malt extract at 50°, the mass rapidly liquefies and the solution acquires a

* v. Fellenberg: "Mitt. Lebensm. Hyg.," 1916, 7, 369.
† Barger: "Ber. deut. chem. Gesells," 1902, 35, 1296.

sweet taste owing to the conversion of starch into maltose; if the latter substance be estimated from time to time, it will be found that the reducing power of the mixture increases rapidly at first until, after about two hours, the amount of maltose present corresponds to about 80 per cent of the starch employed, when practically no further change takes place. The change in the starch paste can also be demonstrated by periodic testing with iodine solution; the blue-black coloration gradually becomes less and less marked until various shades of red are obtained, finally the iodine gives no distinctive coloration. A corresponding fall in the optical activity of the solution can also be observed, but as the activity is still greater than what it should be for maltose alone, it must be concluded that some other substance is formed at the same time as the maltose, and that its reducing power is less but its activity is greater than that of maltose. The amount of this "non-maltose" product of diastatic activity varies directly with the temperature, and increases considerably at the expense of the maltose if the temperature be kept at or above 60°; if to such a product, rich in non-maltose, a fresh quantity of malt extract be added, the non-maltose will be attacked and converted into maltose until the amount present again attains the value 80 per cent, which is the normal maximum; this experiment, which is due to Brown and Morris,* shows that the non-maltose is composed of different constituents, some of which are converted into maltose by diastase more readily than others; moreover experiments have shown that these substances behave differently towards yeast, some being more readily fermentable than others. This non-maltose constituent represents a mixture of the various dextrins mentioned above as having been described by several authors. More recently Maquenne and Roux † and others, carrying on the experiments of Brown and his collaborators, have found that on prolonged action extending over several days, even this non-maltose is slowly attacked by diastase, and a practically theoretical yield, instead of only an 80 per cent yield, can be obtained.

The action of malt on starch accordingly takes place in two stages, of which the first is rapid, being completed in about

* Brown and Morris: "J. Chem. Soc., Lond.," 1885, **47**, 527.
† Maquenne and Roux: "Compt. rend.," 1906, **142**, 124, 1059.

two hours, while the second one is very slow. According to Maquenne and Roux, the first stage corresponds to the hydrolysis of the amylo-cellulose (amylose) and the solution of the amylo-pectin with consequent formation of dextrins; the second or slow stage consists in the hydrolysis of these dextrins into maltose, and they consequently regard amylo-pectin as a maltosane.

It was mentioned above that a larger yield of non-maltose is obtained at higher temperatures, and that this is regarded as a mixture of dextrins, since some of it is readily converted into sugar on adding more diastase, whilst some still remains which resists; this latter is most likely produced from the amylo-pectin and corresponds to the stable dextrin described by Brown and Morris, whereas the easily converted portion is in all probability identical with what Brown and Morris called maltodextrin or amyloin, and may have been produced by a peculiar action of malt on the amylo-cellulose (amylose) constituent of the starch.

*General Properties of Dextrins.*

From what has been said above, it will be seen that the term dextrin comprises a number of substances some of which are not at all well defined. The following may, however, be regarded as approximately representing the characteristics of all substances included in this group:—

1. They are amorphous substances which are readily soluble in water to form gummy solutions, which are used as a substitute for natural gum; they are precipitated from aqueous solutions by the addition of alcohol.

2. Unlike starch inulin and glycogen, dextrin does not give a precipitate with basic lead acetate.

3. As their name implies, they are strongly dextro-rotatory, in which respect they resemble starch.

4. They give either a red colour or no colour at all with iodine.

5. They are not fermentable by yeast alone, but are fermented by a mixture of yeast and diastase acting together, which is no doubt due to their slow hydrolysis in the first place by the diastase and the subsequent fermentation of the maltose so produced.

6. They do not reduce Fehling's solution when pure.

7. They are converted into glucose on hydrolysis with mineral acids.

As has already been mentioned, starch when suddenly heated to about 200° is converted into a substance commercially known as dextrin. The use of starch for stiffening linen depends on some such similar change produced in the starch by the heat of the iron.

Although a great many different dextrins have from time to time been described, comparatively few of them are sufficiently well defined to warrant any description here. The three following, in addition to maltose and isomaltose, were isolated by Lintner and Düll* from the products of the action of malt extract on starch by a long process of fractional precipitation with alcohol.

*Amylo-dextrin.*—This substance, which is regarded by these authors as the chief constituent of soluble starch, is a white powder which is slightly soluble in cold water, but readily in hot. It is strongly dextro-rotatory ($a_D = + 196$), does not reduce Fehling's solution, and gives a blue colour with iodine.

*Erythro-dextrin.*—This is a solid which dissolves readily in water, has a rotatory power of $a_D = + 196°$, and with iodine produces a red-brown colour.

The existence of erythro-dextrin as a chemical entity is, however, disputed by Ost, who says that it is a mixture of achroo-dextrin with starch; an artificial mixture of achroo-dextrin with a half per cent of starch also produces a red colour with iodine.

*Achroo-dextrin.*—This substance is optically active, has the value $a_D = + 192°$, gives no colour with iodine, and has a sweetish taste.

### COMMERCIAL DEXTRIN.

Commercial dextrin is prepared by heating starch to about 230-260°; it is a yellowish-brown powder, while that prepared by acid hydrolysis of starch is an almost colourless solid with a choncoidal fracture, or else a white powder resembling starch. It is composed chiefly of achroo-dextrin mixed with

* Lintner and Düll: "Ber. deut. chem. Gesells.," 1893, **26**, 2533.

varying quantities of erythro-dextrin and glucose. It dissolves in an equal volume of water to give a neutral sticky solution with a faint sweet taste; the solution is strongly dextro-rotatory. Dextrin is insoluble in alcohol and ether.

### GLYCOGEN.

This substance, although one of the most important and widely distributed reserve foods in the animal kingdom, has a restricted distribution in plants. It occurs abundantly in certain Fungi, especially in *Saccharomyces cereviseae*, where it may sometimes form as much as 30 per cent of the dry weight. It has also been described as forming part of the cell-contents in Myxomycetes, Flagellates, and possibly in certain Algae and Cyanophyceae. In the yeast plant the glycogen varies in amount according to the physiological phase of the organism, and, it appears, accumulates and disappears often with great rapidity.

The glycogen appears in the cells of *Saccharomyces* during the early stages of fermentation as minute refractive granules scattered through the protoplasm; after a few hours these granules give place to small vacuoles, which in turn are replaced by one large vacuole, which may occupy the greater space in the cell. According to Harden and Rowland,* this progressive increase in the size of the glycogen-vacuole may result from the formation of some substance, besides carbon dioxide, from the glycogen.

Wager and Peniston† have shown that the amount of glycogen present is correlated with the periodical fluctuations in the fermentative activity.

On adding yeast to the nutrient fluid, e.g., Pasteur's solution, fermentation may start at once, in which case it was found that the cells float and contain very little glycogen. On the other hand, the cells may contain much glycogen and sink to the bottom; in this case fermentation is slow to commence, but gradually increases, and eventually becomes much more active; also the budding is much more extensive as compared with a yeast which contains but little or no glycogen.

* Harden and Rowland: "J. Chem. Soc., Lond.," 1901, **79**, 1234.
† Wager and Peniston: "Ann. Bot.," 1910, **24**, 45.

If healthy brewers' yeast be added to Pasteur's solution the cells, which contain much glycogen, sink to the bottom. After an hour or two the cells begin to rise, and they become distributed throughout the medium after the lapse of four or five hours. The fermentation is now much more active, and the amount of glycogen in the cells less. The next five to fifteen hours is the period of maximum vegetative activity, during which the glycogen disappears; then it slowly reappears, and later on much more rapidly, at which phase there is a marked decrease in budding. At the height of fermentation, or immediately after, the glycogen increases rapidly, and a large number of cells sink to the bottom of the fluid. If the medium be not exhausted, the process may be repeated two or three times.

Although glycogen may be looked upon as a temporary reserve food-matter, for yeast-cells rich in glycogen retain their vitality much longer than those in which there is little or none, the fact that in the spores of species of *Mucor* and in sclerotia glycogen does not appear until growth has commenced, points to the conclusion that in these plants, at any rate, it is not primarily a storage product. Kohl considers that since it is more abundant in *Saccharomyces* during active gemmation, it is not exclusively a reserve substance, but an intermediate product in the formation of alcohol from the sugar.

In the animal kingdom, according to Hoppe-Seyler, glycogen is an invariable constituent of almost all developing cells; it is found also in the muscles and blood, and chiefly in the liver, where it is stored in larger quantities.

It may be remarked that there is little doubt that the glycogen obtained from animal and plant sources are identical.

### *Preparation.*

The following method of obtaining glycogen was devised by Pflüger.* Fresh finely-cut liver is stirred up with water and 60 per cent caustic potash, and heated for two hours; the filtered solution, containing 15 per cent of potash, is then mixed with an equal volume of 96 per cent alcohol, and the

* Pflüger: "Pflüger's Archiv f. Phys.," 1902, **91**, 119, and 1903, **93**, 163.

precipitated glycogen is collected and washed with a mixture of one part of 15 per cent potash with two parts of 96 per cent alcohol; if necessary, the substance may be redissolved and purified in the same way.

Glycogen may also be prepared from yeast, but not in a particularly pure state, in the following manner: A quantity of bakers' yeast, which has been previously well washed with water, is mixed with fine well-cleaned sand and ground very thoroughly in order to rupture the cells. The mixture is then placed in a vessel with about thrice its volume of water and heated for some time, being constantly stirred. The liquid is then filtered off, cooled, and strong alcohol added to the filtrate in order to precipitate the glycogen, which is filtered off. The glycogen so obtained may be purified by redissolving it in water, adding a little acetic acid, and boiling in order to remove any proteins which may not have been removed by the initial heating, filtering, and precipitating with alcohol.

The following method has recently been described by Harden and Young*: The yeast is ground with an equal weight of sand. It is then extracted by boiling with water, and an equal volume of alcohol added to the cooled and filtered liquid. The precipitate formed is collected, washed with 50 per cent alcohol, and is then treated on the boiling water bath with a 60 per cent solution of potassium hydroxide for two hours. The liquid is cooled and poured into an equal volume of water, filtered, and the filtrate precipitated by the addition of two volumes of alcohol. The precipitate is collected, and washed repeatedly with a mixture containing 400 c.c. of water, 100 c.c. of 50 per cent potassium hydroxide, and 500 c.c. of alcohol; it is finally washed with 50 per cent alcohol.

The precipitate is then dissolved in water, and the solution, which is alkaline owing to the difficulty of washing away all the potassium hydroxide, is neutralized with acetic acid, and the glycogen precipitated by the addition of an equal volume of alcohol. By repeatedly dissolving in water, and reprecipitating with alcohol, a preparation may be obtained free from nitrogen and ash, but it still contains yeast-gum,

* Harden and Young: "J. Chem. Soc.," 1912, 101, 1928.

which may be removed by redissolving the crude glycogen in water and saturating with ammonium sulphate. The precipitated glycogen, after being washed with saturated ammonium sulphate, is dissolved in water, and the solution again saturated with ammonium sulphate, the process being repeated three times. The final precipitate is again dissolved, the solution dialysed until free from ammonium sulphate, and the glycogen precipitated with alcohol. For details of the further purification of the glycogen the original paper should be consulted.

### *Properties.*

Pure glycogen is a snow-white amorphous solid. It is readily soluble in hot water, forming an opalescent solution, from which it may be precipitated again by alcohol, provided small quantities of dissolved salts are present; 100 c.c. of a 1 per cent solution when mixed with 200 c.c. of absolute alcohol remain clear, but on adding 0·03–0·05 gram of sodium chloride, an immediate precipitate is formed. Glycogen is strongly dextro-rotatory, $\alpha_D = + 189{\cdot}9^\circ$, and is coloured red to brown by iodine; it does not reduce Fehling's solution, but is broken up by diastase into dextrin and maltose, and by acids into glucose.

### *Identification.*

1. The opalescent appearance of its aqueous solution is characteristic, and is strongly dextro-rotatory.
2. A brown coloration is given with iodine solution (cf. inulin, p. 136).
3. A white precipitate is given with basic lead acetate.
4. It does not reduce Fehling's solution.
5. On boiling with mineral acids, it is converted into dextrose.

### *Estimation.*

This is best effected by heating the aqueous solution for three hours in a boiling water bath with about 2·2 per cent HCl, and then neutralizing and estimating the resulting glucose by means of Fehling's solution; the amount multiplied by 0·9 gives the weight of glycogen.

### PARA-DEXTRANE AND PARA-ISODEXTRANE.

These substances have been isolated from *Boletus edulis* and *Polyporus betulinus* respectively. The former gives a yellow colour with chlorzinc iodide, and the latter a blue when treated with iodine and sulphuric acid. Both give glucose on hydrolysis.

## LEVULOSANES.

### INULIN.

This substance is commonly found as a reserve food-stuff, of the same nature as starch, existing in a state of solution in the cellsap of a number of plants belonging to the natural order Compositæ, e.g. in the tubers of the dahlia and artichoke (*Helianthus tuberosus*), and in the fleshy roots of the chicory (*Cichorium Intibus*). It has also been described as occurring in the following natural orders: Violaceæ, Malpighiaceæ, Droseraceæ, Candolleaceæ, Goodeniaceæ, Campanulaceæ, Lobeliaceæ, Myoporineæ, Liliaceæ, and Amaryllidaceæ; also in some Algæ, e.g. *Neomeris*.

Inulin, or closely allied substances, are not infrequently found in company with starch, especially in some Monocotyledons; and the same peculiarity in its occurrence, as has already been remarked upon in connexion with the occurrence of starch in monocotyledonous plants, obtains (p. 113).

Thus in *Iris pseudacorus* starch is present but not abundant, in *Iris Xiphium* both starch and inulin are present in quantity; *Scilla nutans* has inulin but no starch, while *Scilla sibirica*, and also *Hyacinthus* and *Muscari botryoides* have both starch and inulin. Inulin is not formed as such in the leaves but in the places of storage by the condensation of the sugars synthesized by the leaf.*

It is of interest to find that the nature of the reserve carbohydrates may often be correlated to the habitat of the plant. Parkin† points out that these reserve substances of aquatic plants and of plants inhabiting wet situations take the form of starch, e.g. *Sparganium*, *Alisma*, *Listera*, *Orchis*, and *Schizo-*

* Colin: "Compt. rend.," 1918, **166**, 224, 305.
† Parkin: "Phil. Trans. Roy. Soc., Lond.," B., 1899, **191**, 169.

*stylis*; whereas, on the other hand, inulin, generally associated with sugar, is the characteristic carbohydrate reserve in those Monocotyledons inhabiting dry situations, e.g. *Allium*, *Asphodelus*, *Anthericum*, *Yucca*, *Tritona*, *Iris Xiphium*, etc.

In this connexion* reference must be made to the work of Lidforss, who showed that plants inhabiting wet situations fall into two distinct categories; those like *Elodea*, *Chara* and *Stratiotes*, which hibernate at the bottom of the pond or stream, contain starch but no sugar; while those which live on the banks where their rhizomes, or other organs of storage, pass the winter out of the water, e.g. *Myosotis* and *Menyanthes*, contain sugar during the winter months. In the former case a temperature of $-2^{\circ}$ C. to $-4^{\circ}$ C. is fatal, while in the latter case the death point is about $-7^{\circ}$ C.

This peculiarity also obtains for many arctic plants; Miyake, Wulff, and others have shown that cold, which means physiological dryness, is conducive to sugar production, so that arctic plants frequently exhibit but a small amount of starch, and relatively large quantities of sugar. Stahl has shown that the leaves of mycotrophic plants, which generally show a feeble transpiration, seldom contain starch, its place being taken by glucose. Lidforss also has shown that the winter green vegetation of Sweden is characterized by the absence of starch from the leaves, the mesophyll, in its place, containing relatively large quantities of sugar, and sometimes oil during the winter months. In summer the leaves of these plants contain starch, which, on the advent of winter, is converted into sugar, from which starch is formed on the rise of temperature in the spring.†

Then, again, it is not uncommon to find sugar stored in the periderm of trees and in the leaves of evergreen plants during the winter; starch, however, may be found in the leaves of evergreen trees during the cold season, its presence being due to feeble photosynthesis.

Reference may be made here to the well-known fact that potatoes turn sweet on exposure to cold. This conversion of starch into sugar is most active at $0^{\circ}$ C., and the action decreases with the rise in temperature, so that above $7^{\circ}$ C. no

* See Blackman: "New Phyt.," 1909, **8**, 354.

† See also Maximow: "Ber. deut. bot. Gesells.," 1912, **30**, 52.

sugar is thus produced. Also if the tubers are suddenly subjected to a temperature of − 1° C., no sugar will be produced. The amount of sugar formed is not great, its maximum being about 3 per cent of the wet weight; the limit of the process depends on the concentration of sugar, and, as Czapek has shown, the transformation of the starch may be prevented, on a lowering of the temperature, if the concentration of sugar be sufficient. If these sweet potatoes be exposed to a higher temperature, all the sugar that remains—some has been used up in respiration—is reconverted into starch.

Œcologically these characters are of value to the plant; for if the water of the cell sap be frozen, the salts held in solution become concentrated and will eventually precipitate the soluble proteins. Parkin points out that the presence of inulin * in the cell sap of the parenchymatous tissues would retard the evaporation of water. It is a well-known fact that water in the presence of oil may be much over-cooled before ice-formation takes place, and the freezing point of water in which other substances, e.g. sugar, are dissolved is depressed, and thus the danger arising from the salting out of the proteins is minimized. But, notwithstanding these facts, plants are frequently subjected to temperatures sufficiently low to cause ice to be formed, and as the water is thus withdrawn, the sugar becomes more concentrated until it will also crystallize out. Both these processes generate heat, which may be sufficient in amount to enable the protoplasm to live. And this is, according to Mez and Lidforss, the explanation of the presence of sugar in winter leaves.

At the same time we must be careful not to push such explanations too far, for there are many exceptional cases; thus Ewart has pointed out that *Dicranum* which contains much oil is less resistant than is *Bryum*, and other mosses, in which such substances are absent. The beetroot also is very susceptible to cold, notwithstanding the fact that it contains much sugar; similarly the seeds of the hemp and willow, which contain much oil, are easily killed by desiccation, whereas the oil-containing seeds of the linseed are highly resistant. Such divergent phenomena must depend on the constitution of the protoplasm.

* See also Grafe and Vouk: "Biochem. Zeitsch.," 1913, 56, 249.

Again, oil is a convenient form of reserve food, especially in small organisms and in reproductive bodies, where space is limited and lightness is all-important and it is desirable to store a maximum of potential energy in the minimum of bulk. Finally, as Parkin points out, the nature of the carbohydrate reserve may depend on the kind of sugar transformed; thus, if saccharose be the chief sugar translocated from the leaves, then it might be expected that starch would be produced, on the other hand inulin might be formed if the available sugar for conversion were levulose.

### *Preparation.*

Inulin may be obtained from dahlia tubers, of which it forms from 10-12 per cent, by crushing them and pressing out the liquid; the residue is then boiled up with a little water and some precipitated chalk and filtered again. The two filtrates are then united and once more boiled with chalk in order to neutralize any acids, and while still warm treated with lead acetate until no further precipitate is formed. The filtered solution is then saturated with hydrogen sulphide, filtered, neutralized with ammonia, evaporated to half its bulk and mixed with an equal volume of alcohol. After one or two days, crude inulin may be filtered off; it may be further purified by warming in aqueous solution with animal charcoal, filtering and adding alcohol; the precipitated inulin is then washed with alcohol and ether, and dried over sulphuric acid.

According to Kiliani,* it may also be prepared by boiling crushed dahlia tubers with water and a little chalk, filtering and freezing the filtrate. When the water cools, the precipitate is filtered off, re-dissolved in hot water and frozen out once more. After repeating this process several times, the inulin is washed with methyl alcohol, ethyl alcohol, and finally ether.

### *Characters.*

Pure inulin forms a white starchy tasteless powder of a sphæro-crystalline nature; it swells up and is readily dissolved in hot water, alkalis, etc., and may be recovered from the aqueous

* Kiliani: "Annalen," 1880, 205, 147.

solution by the addition of alcohol, in which it is practically insoluble, or by freezing. Inulin is lævo-rotatory and unlike starch does not give a paste with water, nor does it give a blue colour when treated with iodine. Diastase has no effect upon it; it may, however, be hydrolysed by the ferment inulase, or by mineral acids, by which reagents it is converted into levulose. The low osmotic pressure which solutions of inulin exert suggests a large molecule, but its molecular structure appears to be less complex than that of starch. The relation between inulin and levulose is much the same as that existing between starch and glucose.

*Identification.*

In many plants the presence of inulin is indicated by the well-known sphæro-crystals which are obtained on steeping the fresh tissues for some time in strong alcohol; this deposition is not, however, always so characteristic; thus in Monocotyledons the inulin is frequently found, after treatment with alcohol, in amorphous masses. The sphæro-crystals and the amorphous concretions of inulin are readily soluble in warm water, and thus may be distinguished from calcium phosphate which may occur in cells in shapes similar to those of inulin. These two substances may be further recognized by the fact that sulphuric acid completely dissolves inulin, whereas it forms with calcium phosphate insoluble calcium sulphate. The following tests also may be performed.

1. Green's Test.—Sections of the material, which have been soaked for some time in absolute alcohol, are treated with a saturated solution of orcin in strong alcohol, and then boiled in hydrochloric acid. The masses of inulin disappear and a red colour results. If phloroglucin be substituted for the orcin, the resulting coloration will be reddish-brown.

2. Molisch's Test.—The sections are treated with a 10 per cent alcoholic solution of $\alpha$ naphthol, then a few drops of strong sulphuric acid are added and the preparation warmed. A deep violet coloration ensues, and the inulin is dissolved.

These colour reactions are indicative of the formation of sugar by the hydrolysis of the inulin by the acids employed in the tests; it is therefore important, before employing these reactions, to make sure that no free sugars are present in the

material to be examined, and to wash the preparations thoroughly with alcohol in order to remove them.

Since inulin does not reduce Fehling's solution, this reagent may be employed to ascertain whether any reducing sugars are present in the material before employing the above tests for inulin.

The following reactions may be carried out with a solution of inulin.

3. The addition of iodine solution gives with inulin a brownish coloration. Since the solution of iodine is itself brown, this test must be performed very critically. The following method may be employed: dilute the solution of iodine with water until it is a light brown colour, fill two test tubes with this solution and add to one a drop of the inulin solution; now compare the colour of the contents of the two test tubes.

This same reaction is also given by glycogen, when the same procedure may be followed.

4. Basic lead acetate gives with inulin, and also with glycogen, a white precipitate. This test may be used to distinguish inulin and glycogen from dextrin, which does not give a precipitate with this reagent.

5. Inulin is precipitated from solution by alcohol.

6. Hydrolyse with mineral acid and test for levulose.

There is as yet no very accurate method for the estimation of inulin. Dragendorff* recommends precipitating the inulin from an aqueous extract and then determining the amount of levulose which is produced on hydrolysis.

### INULIN-LIKE SUBSTANCES.

Attention may now be drawn to substances similar to inulin which occur in various plants. The chief of these are:—

Graminin in *Agrostis*, *Festuca*, *Trisetum* and other grasses.

Irisin in *Iris pseudacorus*.

Phlein in *Phleum pratense* and *Phalaris arundinacea*.

Sinistrin in *Scilla maritima*.

Triticin in *Triticum repens*, *Dracæna australis* and *Dracæna rubra*.

* Dragendorff: "Jahres. Fortsch. d. Chem.," 1872, 929.

All these compounds have the same formula, $6(C_6H_{10}O_5 + H_2O)$, and possess the same characteristics; they are lævo-rotatory, yield fructose on hydrolysis, and are fairly soluble in cold water. The majority are difficult to crystallize, and their solutions yield a gum-like substance on evaporation. It is possible that some, at any rate, of these substances may bear the same relation to inulin as dextrin does to starch.

## MANNOSANES.

### MANNANE.

The seeds of many plants contain reserve carbohydrate which is generally referred to as reserve cellulose, hemi-cellulose, or para-galactane substances. These materials are often indistinguishable from true cellulose by the microchemical means at our disposal. They may, however, be distinguished by the products of their hydrolysis; thus they form glucose, together with other substances, on treatment with hot dilute hydrochloric or sulphuric acid, whereas ordinary cellulose does not. Also they are dissolved by dilute alkalis, and by cuprammonia after a *brief* treatment with hot dilute hydrochloric acid. These reserve celluloses contain mannanes and galactanes.

Mannane serves in the same way as starch as a reserve food-supply to a very large number of different plants. It may be regarded as an anhydride of mannose, since it yields this substance on hydrolysis; it occurs either alone or united to anhydrides of other sugars, such as glucose, galactose, pentose, etc., in a great many different forms.

A fairly pure specimen of mannane can be obtained from yeast by a somewhat elaborate method devised by Hessenland.* It is a white amorphous substance, which is somewhat soluble in water and swells up in dissolving; it is insoluble in alcohol but readily soluble in alkali, and is strongly dextro-rotatory $a_D = + 283{\cdot}7\text{-}287{\cdot}6°$.

Mannane occurs in salep mucilage, and has been extracted by Ritthausen† and Effront‡ and others from wheat and barley. Mannanes are also found in *Penicillium glaucum*,

* Hessenland: "Z. d. Vereins d. Deut. Zuckerind.," 1892, **42**, 671.

† Ritthausen: "J. prakt. Chem.," 1867, **102**, 321, and "Chem. Zeit.," 1897 **21**, 717.

‡ Effront: "Compt. rend.," 1897, **125**, 38, 116.

ergot, in the roots of several plants such as asparagus, chicory *Helianthus* and *Taraxacum;* also in the wood and leaves of many trees, such as lime, chestnut, apple, mulberry, certain Oleaceæ and conifers; the so-called reserve celluloses and hemi-celluloses contained in seeds of Palmaceæ, Liliaceæ, elder, cedar and larch, and many other plants, are also very rich in mannanes.

### PARAMANNANE.

Paramannane is a variety of mannane which is characterized by being much more resistant to hydrolysis; this substance, which is contained in coffee beans, is only slightly acted on by hot dilute mineral acids, potassium chlorate and hydrochloric acid, but dissolves in a concentrated hydrochloric acid solution of zinc chloride. It is accordingly frequently classed as a mannose-cellulose.

### CARUBIN OR SECALANE.

Carubin* is the name given to a substance occurring in the seeds of *Ceratonia siliqua*, and in various cereals such as rye and barley. In its characters it closely resembles mannane, and by some authors is regarded as identical with it; when dry, it is a spongy friable substance which swells upon the addition of water. It is soluble in cold water and is optically inactive. Its sugar is fermentable and non-crystalline.

## GALACTOSANES.

### GALACTANE.

Exactly analogous to the mannanes are the galactanes, which may be looked upon as anhydrides of galactose. They occur in a great variety of different forms, some of which are readily hydrolysed by warming with alkali, while others are very resistant even towards boiling alkali. Four galactanes have been described, which are distinguished by the prefixes $\alpha$–, $\beta$–, $\gamma$– and $\delta$–; they are all amorphous substances which dissolve with difficulty in water, and on hydrolysis yield galactose.

* Effront: "Compt. rend.," 1897, 124, 200, and 125, 116 and 309.

### PARAGALACTANE.

Paragalactane is a substance which is better termed paragalacto-arabane, since on hydrolysis by weak mineral acids it yields a mixture of galactose and arabinose. It occurs in the cell walls of the cotyledons of many plants, e.g., *Lupinus luteus* and other species, *Phœnix dactylifera*, *Cocos nucifera*, and other palms, *Soja hispanica* and *Coffea arabica*, where it forms a reserve food-material which is digested on germination.

Paragalactane is a white solid which is insoluble in water and cuprammonia; it dissolves in hot potash. On heating with nitric acid it is oxidized to mucic acid. Microchemically it may be identified by its insolubility in the reagents mentioned, and also by the fact that with phloroglucin and hydrochloric acid it gives a red coloration on warming. No colour is given in the cold.

Its association with cellulose prevents the latter exhibiting some of its reactions; thus the cellulose is unacted upon by cuprammonia unless the paragalactane be removed; this may be done by boiling in dilute hydrochloric acid.

### AMYLOID.

Amyloid * is the name given to a substance occurring in the seeds of pæonies and certain cresses,† which yields on hydrolysis with dilute sulphuric acid a mixture of galactose, glucose, and xylose. It is a colourless substance, and is insoluble in cold water, but swells up into a slimy mass in hot water; it is soluble in cuprammonia solution. Amyloid does not reduce Fehling's solution, but is oxidized by nitric acid to mucic and trihydroxy-glutaric acids. It gives a blue colour with iodine.

## GUMS.

The natural gums were formerly thought to be carbohydrates of the general formula $(C_6H_{10}O_5)_n$; the researches of O'Sullivan, however, have shown that they are not simple carbohydrates, but are rather substances of a glucosidal nature, since on hydrolysis they give rise to sugars mixed with complex acids of high molecular weight. The gums themselves retain

* Cf. footnote, p. 152.

† Winterstein: "Z. physiol. Chem.," 1893, 17, 353.

the character of acids, and it would appear that the molecule of a gum is composed of a number of sugar residues grouped around a nucleus acid in such a way as to leave the acid group exposed.

The gums are translucent amorphous substances, some of which dissolve in water completely, giving a sticky solution, while others merely swell up in water and form a sort of jelly; they are all insoluble in alcohol.

The natural gums must be distinguished from starch gum or dextrin, which is an artificial product obtained from starch, by the following characteristics:—

1. Solutions of natural gums are lævo-rotatory, whereas those of dextrin are dextro-rotatory.

2. Basic lead acetate precipitates natural gums from solution, but has no action on dextrin.

3. Natural gums on hydrolysis yield chiefly galactose and pentoses such as arabinose or xylose, whereas dextrin yields glucose only.

The hydrolysis of gums takes a long time to complete—from eighteen to twenty-four hours—whereas dextrin is easily hydrolysed.

4. On oxidation with nitric acid, natural gums yield chiefly mucic acid ($C_6H_{10}O_8$) together with some saccharic ($C_6H_{10}O_8$) and oxalic ($C_2H_2O_4$) acids, whereas dextrin yields chiefly oxalic acid together with a small quantity of saccharic and tartaric ($C_4H_6O_6$) acids.

As they occur in nature, the true gums are mostly combined with potassium, calcium, or magnesium in the form of salts, from which the true carbohydrate can be isolated by the action of a stronger acid.

The classification of gums is, for want of more accurate knowledge, based chiefly on their solubility in water:

(*a*) Gums, such as arabin, which are completely soluble.

(*b*) Gums which are partially soluble, such as cerasin and bassorin.

(*c*) Mucilages and pectic bodies which merely swell up with water to form a jelly.

The classification, however, is by no means rigid, many natural gums being composed of mixtures of several kinds of gums.

In the separation of gums from the tissues of the plant advantage is taken of their solubility in water; it is found in practice, however, that in many cases mere maceration in water does not remove all the gum present; Dragendorff found that much more arabic acid could be extracted after the material had been treated with an alcoholic solution of tartaric acid.

### *Microchemical Reactions.*

Microchemically, gum and mucilage may be recognized by their solubility and swelling respectively in water. Both are insoluble in alcohol and ether. With other reagents the results differ in different examples. Thus with iodine either a blue or a yellow colour may result, while in other cases the blue coloration is only obtained after treatment with chlorzinc iodide or sulphuric acid and iodine. Then again different degrees of solubility are found to obtain on treatment with cuprammonia. Many of these substances stain well with corallin soda, and they also, especially the mucilages, show a great avidity for stains such as aniline blue and aniline violet.

### GUM ARABIC.

This substance is a mixture of calcium, magnesium, and potassium salts of a weak acid of unknown constitution, to which earlier writers gave the name of arabic acid or arabin. O'Sullivan, however, applied the term arabic acid to a substance of the formula $C_{23}H_{38}O_{22}$, which he regarded as the nucleus acid around which a number of sugar residues are grouped; by hydrolysis under varying conditions, it is possible to split off successive sugar residues with the formation of acids of gradually decreasing molecular weight, until finally the nucleus acid free from all carbohydrate residues remains, and it is this acid that he calls arabic acid; the natural gum itself would, according to him, be a diarabinan-tetragalactan-arabic acid of the formula $2C_{10}H_{10}O_8$, $4C_{12}H_{20}O_{11}$, $C_{23}H_{30}O_{18}$, which is combined with the calcium, magnesium, and potassium. The arabic acid of the earlier authors, which is the acid set free from the natural gum by the removal of the calcium, magnesium, and potassium, may be prepared by acidifying a

concentrated aqueous solution of gum arabic with hydrochloric acid, and adding alcohol. The pure substance is a white amorphous glassy mass which dissolves in water to give a lævo-rotatory solution. Ten per cent sulphuric acid converts this arabic acid into metarabic acid, which swells up in water, but does not dissolve.

*Reactions.*

Solutions in water (10 per cent) of arabic acid and other varieties of gum arabic give, according to Masing,* certain more or less definite reactions.

1. They are not precipitated by (*a*) a cold saturated solution of copper acetate; (*b*) 10 per cent solution of lead acetate; (*c*) solution of ferric chloride (sp. gr. 1·2).

2. A five per cent solution of silicate of potash produces a cloudiness or a precipitate which is partially or wholly soluble on adding an excess. Arabic acid either does not respond to this reagent, or merely gives a slight turbidity, and the same applies to the gums obtained from certain species of *Cactus*, *Albizzia*, *Acacia catechu*, *Acacia leucophlœa* and other plants.

3. Stannate of potash gives similar reactions, and in the case of arabic acid produces a precipitate which is soluble in excess.

4. A solution of neutral sulphate of aluminium (10 per cent) generally gives a precipitate which is, in many cases, soluble in potash.

5. Basic lead acetate yields a precipitate which is entirely or partially soluble in excess.

GUM TRAGACANTH.

This gum occurs in species of *Astragalus*, and consists of about 8-10 per cent of soluble calcium, magnesium, and potassium salts, together with about 60-70 per cent of insoluble salts, which only swell up in water to a jelly. The water soluble portion is said to contain a substance, polyarabinou-trigalactan-geddic acid, which on hydrolysis breaks up into arabinose, galactose, and geddic acid, an isomer of

* Masing: "Archiv d. Pharm.," 1879, [3], 15, 216; 1880, 17, 34, 41; "Year Book of Pharmacy," 1881, 191.

arabic acid. The part soluble in water, when treated with baryta water, gives two isomeric tragacanthan-xylan-bassoric acids, which on hydrolysis yield a pentose sugar tragacanthose, xylose, and bassoric acid $C_{14}H_{20}O_{13}$.

### WOOD GUM AND CERASIN OR CHERRY GUM.

These are pentosanes which occur in the wood of trees such as beech, oak, cherry, ash, etc.; they are extracted with alkaline solutions, and on hydrolysis yield xylose.

### WOUND GUM.

A gum-like substance, termed wound gum, is frequently found in the tracheæ of plants, in the immediate neighbourhood of wounds, and stopping up the lumina; it is secreted by the surrounding living cells. Wound gum does not swell in water, and is insoluble in sulphuric acid and in caustic soda. On oxidation with nitric acid it yields both mucic and oxalic acids, and it responds to lignin tests; e.g., on treatment with phloroglucinol and hydrochloric acid a bright red coloration results.

### MUCILAGE.

The term mucilage is applied to those substances which with water produce a slimy liquid. Mucilage is widely distributed, and occurs in all or nearly all classes of plants. Mucilage-secreting hairs, or comparable structures, occur in various Muscineae, Filices, and especially in the Phanerogams; mucilage sacs or canals are found in certain Muscineæ, e.g., *Anthoceros*, Marattiaceæ, some Cycadaceæ, and Phanerogams; finally, the external walls of plants may be generally mucilaginous; e.g., in very many Algæ, the hibernaculæ of some aquatic Phanerogams, like *Utricularia* and *Myriophyllum*, and finally in the coats of seeds and fruits, such as *Lepidium* and *Sterculia scaphigera* respectively, in which cases the superficial cell walls are mucilaginous. Mucilage is not infrequently associated with other substances; thus in the case of mucilage-secreting hairs, it is sometimes associated with tannin, and in many plants, especially in the mucilage sacs of many Monocotyledons, calcium oxalate is found.

The constitution of mucilages is as yet unknown; they are, however, related pretty closely both to cellulose and to arabin. In fact, by some authors they are regarded as decomposition products of cellulose, produced either by overnutrition of certain cells or by bacterial action;* according to Wiesner, all gums are produced by a diastatic ferment acting on cellulose; although it is not possible to express any definite views on the subject, it would appear not improbable that in many cases the formation of gums and gum-like substances in the plant is a morbid condition. Mohl was able to show in the case of tragacanth gum that this substance was produced by the metamorphosis of the cells of the medullary rays.

That mucilages are not all of the same constitution is shown by the fact that the mucilaginous substance obtained from *Fucus* (caragheen mucilage) on hydrolysis with dilute sulphuric acid yields galactose, while salep mucilage, obtained from *Orchis Morio*, on a similar treatment yields mannose.

### *Function.*

Mucilage, when it is a definitely secreted product or of a definite and constant occurrence in a plant, may perform several functions, but how far these are primary functions cannot yet be stated.

When it occurs in tubers, as in the Orchidaceæ, mucilage is generally looked upon as a reserve food-material; it may serve as a check against too rapid transpiration, especially when produced in connexion with developing organs, such as vegetative buds, young leaves, in the epidermis of mature leaves, the sporangia of Cryptogams, etc.; in the case of aquatic plants, such as Algæ, the hibernaculæ of *Myriophyllum*, etc., its presence may prevent a too rapid diffusion; the calcareous incrustation of certain Algæ, e.g., *Neomeris dumetosa*, is dependent on the presence of mucilage; mucilage provides a water-storage mechanism in plants subjected to xerophytic conditions, e.g., *Cassia obovata*, *Malva parviflora*, *Theobroma cacao*, and *Pterocarpus saxatilis*; finally, it may be an important aid in connexion with seed-dispersal and germination, as in some species of *Salvia* and *Lepidium*.

* See Greig Smith: "J. Soc. Chem. Ind.," 1904, 105, 972.

Related to the gums and mucilages are the substances known as galactosanes occurring in the seeds of Leguminosæ (*Lupinus*, *Medicago*, etc.); wood gum or xylane, occurring in wood, etc. etc. These substances have already been mentioned in connexion with the sugars which they give rise to on hydrolysis.

## PECTIC BODIES.

Many succulent fruits, such as pears, apples, gooseberries, and currants, and also fleshy roots, such as carrots, beetroots, etc., contain, together with the cellulose in the cell walls of parenchymatous elements, a substance which is soluble in water, but whose aqueous solution gelatinizes spontaneously on cooling;* this substance, which is probably the cause of concentrated aqueous extracts of fruit gelatinizing, is known as pectin.

According to Frémy,† the hardness of unripe fruit is due to the presence of pectose, an insoluble substance, which is deposited in the cell walls; as the fruit ripens the pectose undergoes a variety of changes, and is ultimately converted into pectin, the soluble substance capable of gelatinizing.

According to Schryver and Haynes,‡ on the other hand, pectin has its origin in pectinogen, a water soluble substance which may be extracted from fruit with 0·5 per cent ammonium oxalate. An alkaline solution of pectinogen at ordinary temperatures is converted into pectin, which has acid characters, and is precipitated by acids from its alkaline solution as a gel insoluble in water.

Under the action of an enzyme pectase contained in the plant, pectin is coagulated; this change was first studied by Frémy, and later by Bourquelot and Hérissey;§ according to Duclaux‖ and others, the coagulation is dependent on the presence of calcium salts, and will take place even in the absence of the enzyme.

* MacNair: "J. Phys. Chem.," 1916, **20**, 633.

† Frémy: "J. Pharm. et Chim.," 1840, **26**, 368.

‡ Schryver and Haynes: "Biochem. Journ.," 1916, **10**, 539.

§ Bourquelot and Hérissey: "J. Pharm. et Chim.," 1898, [6], **8**, 145; 1899, [6], **9**, 563, and **10**, 5. Also Verdon: "J. Pharm. Chem.." 1912, **5**, 347.

‖ Duclaux: "Traité de Microbiologie," 1899, II, 336, and Goyaud: "Compt rend.," 1902, **135**, 537.

Comparatively little is known about the chemistry of these substances; at one time there was even some doubt as to whether they were really carbohydrates, since the ratio of hydrogen to oxygen seemed to be less than that required for compounds belonging to this group. Analyses by Tromp de Haas and Tollens,* however, agreed fairly well either for the formula $(C_6H_{10}O_5)_n$ or $2C_6H_{10}O_5 . H_2O$. On the other hand, Schryver and Haynes find that the analysis agrees with the formula $C_{17}H_{24}O_{16}$.

Pectinogen distilled with hydrochloric acid yields furfural in a quantity which indicates that one pentose group is contained in each complex of 17 carbon atoms.

There is evidence in these substances of acid groups combined with metallic elements as in gum arabic;† by boiling pectose with dilute acids or caustic alkalis, different substances are produced, such as pectin, parapectin, metapectin, pectic acid—which is combined with bases, such as calcium, and forms the middle lamella of cell walls—parapectic acid and parapectosic acid, some of which are soluble in water, while others, such as pectin, swell up in water and gelatinize. The final product of these changes, namely, metapectic acid, is readily soluble in water; it appears to be closely related to, or identical with, arabic acid, and on hydrolysis with dilute sulphuric acid gives arabinose.

This view receives confirmation from the work of Bourquelot and Hérissey, who discovered an enzyme occurring in malt, which is not identical with diastase, and which is capable of hydrolysing pectose to a reducing sugar, namely, arabinose. This enzyme, to which they gave the name pectinase, acts both on unaltered and on coagulated pectic bodies, but, conversely, the coagulating enzyme pectase is without action on pectic bodies which have been previously hydrolysed by pectinase.

Collectively, the pectic substances are not reducing bodies, and are insoluble in cuprammonia. They give precipitates with metallic salts, and are likewise thrown down by the sulphates of magnesium, ammonium, or sodium, or by alcohol.

* Tromp de Haas and Tollens: "Annalen," 1895, **286**, 278.
† Ehrlich: "Chem. Zeit.," 1917, **41**, 197.

To summarise, it appears that pectic bodies are compounds of various carbohydrates with acid groups of undetermined constitution which form molecular complexes that may be entirely resolved by the continued action of simple hydrolytic agents.*

*Microchemical Reactions.*

The fact that these pectic substances are akin to cellulose, and occur in conjunction with it, renders its identification by microchemical means somewhat difficult. Mangin† more particularly has investigated these matters, and gives the following methods :—

1. Methylene blue, Bismarck brown, and fuchsin stain pectic substances, lignified and suberized walls, but not pure cellulose. If sections thus stained are treated with alcohol, glycerine, or dilute acids, the lignified or suberized walls retain their coloration, whilst the pectic substances are decolorized with rapidity.

2. Crocein and nigrosin stain lignified and suberized walls, but do not stain pectic compounds.

3. Crocein, naphthol black, and orseille red stain pure cellulose, but do not stain pectic substances ; similarly, pectic compounds are unstained by congo-red and azo-blue, whilst cellulose and callose are.

4. The middle lamella, which consists of compounds of pectic acid, may be differentiated from the other pectic substances which are mixed with the cellulose of the cell walls by the following method : A thin section is placed in a 20-25 per cent solution of hydrochloric acid in alcohol for twenty-four hours ; the section is then washed with water and treated with methylene-blue or phenosafranin. The middle lamella stains much more deeply than the rest of the wall.

5. If, after the above treatment with acid alcohol, the section be washed in a 10 per cent solution of ammonia, it is found that the cells separate with ease one from the other. According to Mangin, the combined pectic acid is freed from its bases by the treatment with acid alcohol, and is then dissolved by the ammonia. A recombination of the pectic acid may be

* Cross and Bevan : "Cellulose," London, 1918. See also v. Fellenberg : "Biochem. Zeitsch.," 1918, 85, 118.

† Mangin : "Compt. rend.," 1889, 109, 579 ; 1890, 110, 295, 644.

brought about by treatment with baryta water, and after this process the cells will not separate one from the other.

6. The cellulose may be separated in the following manner: A thin section is treated with cuprammonia for twenty-four hours; it is then washed, first with water, and, finally, with 2 per cent solution of acetic acid. The cellulose is thus dissolved and fills the cells and intercellular spaces. On treatment with chlorzinc iodide the middle lamella gives either no colour reaction or turns a pale yellow, while the cellulose gives the familiar blue reaction; the membrane stains very deeply with safranin or methylene blue, and is easily soluble in a solution of ammonium oxalate.

REFERENCES.

O'Sullivan: "J. Chem. Soc., Lond.," 1884, 45, 41; 1890, 57, 59; 1891, 59, 1029; 1901, 79, 1164.

H. H. Robinson: "Brit. Ass. Reports, York," 1906, 227.

Haynes: "Biochem. Journ.," 1914, 8, 553.

## CELLULOSE.

The term cellulose should be taken in general to connote a group of substances rather than a single chemical compound; used in this generic sense, it comprises a number of substances of somewhat different origin and somewhat different characters, whose chief common properties are their physiological origin and their function in forming the basis of the material which is isolated by the protoplasm of the living cell for the purpose of forming the wall or periphery of that cell. Though met with chiefly in the vegetable kingdom, its occurrence in the animal kingdom is not unknown, since a substance described as Tunicin, said to be identical with cellulose, has been found in the cell walls of certain Tunicates and insects. In the course of time the cellulose originally formed is altered by the addition to it of various secondary products known as encrusting substances; thus the process of lignification consists in the conversion of cellulose into ligno-cellulose; accompanying this change is a gradual disappearance of the protoplasm. Thus the protoplasm within the cell produces a number of different substances which are deposited in the cell wall, the nature and properties of the resulting fibre depending, of course, on the nature of these substances.

## CLASSIFICATION OF CELLULOSES.

The naturally occurring celluloses may be divided into the following groups:—

I. Typical or Normal Celluloses of the Cotton Type.—These are exemplified by the cellulose obtained from cotton, flax, hemp, etc.

II. Compound Celluloses of the Wood Cellulose, Jute and Cereal Grass Types.—The natural celluloses occurring in jute, cereal straws, esparto grass, etc., consist of some form of cellulose combined with a non-cellulose constituent, which may be either of the nature of lignin in the case of lignocelluloses, or a pectic or gummy substance in the case of pectocelluloses, or a fatty substance in the case of adipocelluloses. This group may therefore be subdivided into—

(*a*) Lignocelluloses, e.g. jute fibre.
(*b*) Pectocelluloses, e.g. flax.
(*c*) Adipo- or Cuto-celluloses, e.g. cork.

III. Hemi-, Pseudo- or Reserve Celluloses.—This is a somewhat heterogeneous collection of substances which differ structurally from the fibrous celluloses, and occur in the cell walls of the seeds of various plants; such as *Coffea arabica*, *Soja hispida*, *Lupinus luteus*, *Cocos nucifera*, *Tropæolum majus*, *Impatiens balsamifera*, *Pæonia officinalis*, and in peas and beans; celluloses of this type are much more easily hydrolysed than other celluloses, and give rise to various sugars, such as mannose, galactose and pentose. For this reason they may be regarded as anhydrides of these sugars, and are therefore treated under the heading of mannosanes (p. 137), galactosanes (p. 138), and pentosanes (pp. 70, 143).

In this group of celluloses are also included those which, according to the researches of Brown and Morris, are dissolved by the enzymes secreted by the germinating seed; these are sometimes referred to as reserve cellulose, though the name seems ill-chosen, inasmuch as they would not appear always to function as reserve material.

One of the richest sources of cellulose in nature is the cotton plant. The following table, taken from Bowman,* represents approximately the composition of cotton fibre from various sources.

* Bowman: "The Structure of the Cotton Fibre," London, 1908, p. 147.

| Source of Cotton. | Surat. | American. | Egyptian. |
|---|---|---|---|
| | Per cent. | Per cent. | Per cent. |
| Cellulose | 91·35 | 91·00 | 90·8 |
| Wax, oil, and fat | ·40 | ·35 | ·42 |
| Protoplasm and derivatives (Pectose) | ·53 | ·53 | ·68 |
| Mineral matter, i.e., salts of K, Na, Ca, Mg, Fe, and Al | ·22 | ·12 | ·25 |
| Water | 7·50 | 8·00 | 7·85 |

Such cellulose, however, in its native condition is not in a pure state, being in more or less intimate chemical union with other substances, such as pectic bodies, lignocellulose and colouring matters from which it has to be freed by a series of successive chemical treatments before the pure cellulose can be isolated.

The chemical treatments referred to are as follows:—

1. Alkaline hydrolysis, which consists in boiling the fibres with 1-2 per cent caustic potash, and washing to remove the pectic bodies.

2. Exposure of the washed fibres to bromine or chlorine at the ordinary temperature; by this process the lignone complex of the lignocellulose is destroyed.

3. A second alkaline hydrolysis with sodium sulphite, carbonate or hydrate.

The cellulose is thus isolated in a very pure state.

### CHARACTERISTICS AND PROPERTIES OF NORMAL CELLULOSE.

In describing the chemical properties of cellulose, the cellulose isolated as above described from the fibre substance of cotton is chosen as typical.

Pure cellulose is a white hygroscopic substance, which absorbs about 6-12 per cent of water, which it loses again when heated to 100°; it is insoluble in water at ordinary pressure, but when heated with water in sealed vessels at 500° F., it is dissolved completely with decomposition.

### SOLUBILITY OF CELLULOSE.

Cellulose is insoluble in all ordinary solvents, but when treated with zinc chloride in the presence of water, it is con-

verted into a gelatinous hydrate which, after prolonged treatment, goes into solution.

A solution of six parts of zinc chloride in ten parts of water heated to 60-100° is thoroughly stirred up with one part of cellulose, and then digested for some time at a gentle heat. When the cellulose is gelatinized, its solution is completed by heating over a boiling water bath, and adding water from time to time to replace that lost by evaporation.

Two other salt solutions are known which dissolve cellulose:—

(*a*) *Zinc chloride and hydrochloric acid.*—A solution of zinc chloride in twice its weight of hydrochloric acid dissolves cellulose rapidly in the cold.

(*b*) *Ammoniacal cupric oxide* (*Schweitzer's Reagent*).—The solution is prepared by adding ammonium chloride and then excess of sodium hydrate to a solution of a cupric salt; the blue precipitate so obtained is then washed, pressed on a cloth filter, and dissolved in 0·92 ammonia. Cellulose dissolves in this solvent and on the addition of acid is reprecipitated; this fact is made use of in the preparation of artificial silk.

### ACTION OF VARIOUS CHEMICALS ON CELLULOSE.

1. *Alkalis.*—Solutions of caustic soda of 1 to 2 per cent strength have no action on cellulose at temperatures considerably above 100°. Solutions containing 10 per cent have a curious effect on cotton fibre, causing it to thicken and become more cylindrical, and destroying the central canal. This phenomenon was first made use of technically by Mercer, who found that by this means cotton could be made to acquire a gloss resembling that of silk, since the fibre becomes translucent during the contraction.

When fused at 200-300° with a mixture of sodium and potassium hydroxides, cellulose undergoes complete decomposition with the formation of oxalic and acetic acids.

The so-called alkali cellulose obtained by mercerizing cellulose with about 15 per cent caustic soda reacts with carbon disulphide to form xanthogenates;* these compounds are used in the manufacture of viscose (see below).

* Cross, Bevan and Beadle: "Ber. deut. chem. Gesells.," 1893, **26**, 1090; and Cross and Bevan: "Ber. deut. chem. Gesells.," 1901, **34**, 1513.

2. *Acids.*—Nitric acid (sp. gr. 1·25) at 180° converts cellulose into *oxycellulose*, a substance of a weak acidic character, which reduces Fehling's solution (see below under oxidizing agents). Concentrated nitric acid, or a mixture of this acid with concentrated sulphuric acid, converts cellulose into nitrates, the composition of which varies with the conditions of the experiment; di-, tri-, tetra-, penta- and hexa-nitrates,* which are of considerable technical importance, are known. If dilute sulphuric acid is allowed to act for some hours at 100° C. on cotton, it does not alter the structure of the fibre, but makes it friable. This was at one time thought to be due to the formation of a definite substance, hydrocellulose, but, according to Stern,† the elementary composition of the cellulose is not altered; the friability is explained by the fact that certain portions of the fibre are more easily attacked than others, and when these are converted into soluble products, the whole fibre falls to pieces.

Concentrated sulphuric acid dissolves cellulose, gradually converting it into dextrin and ultimately into dextrose. If the solution, as soon as made, is diluted with water, a gelatinous hydrate is precipitated; ‡ this substance is known as amyloid, § since it resembles starch in giving a blue colour with iodine. The same substance is formed by the action of chlorzinc iodide, the reaction being used as a test for cellulose.

When boiled with 40 per cent hydrochloric, or 73 per cent sulphuric acid, cellulose is hydrolysed with formation of esters of polysaccharoses,‖ which contain acidic hydroxyl groups. The claim made by Willstätter and Zechmeister¶ that cellulose can be *quantitatively* hydrolysed to glucose is therefore inadmissible.

The combined action of glacial acetic acid and acetic

* See footnote, p. 159.

† Stern: "J. Chem. Soc.," 1904, **84**, 336.

‡ This reaction is made use of in the preparation of parchment paper. For this purpose paper is rapidly drawn through a mixture of four parts of concentrated sulphuric acid with one part of water; the paper is then thoroughly washed with water until it is free from acid.

§ This substance must not be confused with a compound of the same name which occurs naturally in several plants (cf. p. 139).

‖ Cunningham: "J. Chem. Soc.," 1918, **113**, 173.

¶ Willstätter and Zechmeister: "Ber. deut. chem. Gesells.," 1913, **46**, 2401.

anhydride in the presence of concentrated sulphuric acid or zinc chloride converts cellulose into *acetyl cellulose*, which is insoluble in water but soluble in several organic solvents. Acetyl cellulose is also used in the manufacture of artificial silk.

Cellobiose,* $C_{12}H_{22}O_{11}$, is a disaccharide obtained in the form of its acetate by acting on cellulose with acetic anhydride and concentrated sulphuric acid. It stands in the same relation to cellulose as does maltose to starch; since cellulose and starch yield different disaccharides on hydrolysis, it would appear that these two substances are fundamentally different and that cellulose is not a higher polymer of starch.

Cellobiose reduces Fehling's solution and gives an osazone, m.p. 208-210°.

3. *Oxidizing Agents.*—Dilute solutions of alkaline hypochlorites have very little action on typical cellulose, and can therefore be employed for bleaching this material; with concentrated solutions of hypochlorites, however, a general decomposition ensues. As already mentioned, nitric acid (sp. gr. 1·25) at 180° converts cellulose into a series of oxidation products known as oxycellulose, and similar substances are produced by the action of other oxidizing agents, such as chromic acid, potassium chlorate, and hydrochloric acid, etc. The nature of these oxycelluloses differs somewhat according to their mode of formation, but in general they are characterized by the fact that they yield a relatively large amount of furfurol on boiling with hydrochloric acid; they are hydrolized by boiling with milk of lime into isosaccharic and dioxybutyric acids; they also reduce Fehling's solution, and are dyed by basic dyes, such as methylene blue.

The fact that the cellulose obtained from esparto grass and cereal straws resembles oxycellulose, in yielding a considerable proportion of furfurol on boiling with hydrochloric acid, leads to the idea that cellulose from these sources contains oxycellulose, but whether or not such oxycelluloses are actually pre-existent in the plant fibre has not yet been definitely established (see Lignocelluloses).

Oxygen containing 2 per cent of ozone at once attacks dry cotton with the formation of a cellulose peroxide † and an

* Skraup and König: "Ber. deut. chem. Gesells.," 1901, **34**, 1115; Schliemann: "Annalen," 1911, **378**, 366.

† Dorée: "J. Chem. Soc.," 1913, **103**, 1347.

acid substance; the latter, when boiled with water dissolves, leaving a neutral product which resembles a typical aldehydic oxycellulose. This is regarded as being due to the oxidation of an alcoholic group into cellulose molecule (see formulæ, p. 56).

4. *Action of Ferments.*—It has been shown by Brown and Morris, in the case of malt, that the cell wall of the endosperm cells which contain nutrient material are broken down by a cellulose-dissolving ferment, a cyto-hydrolyst, before the embryo can procure the food-stuff contained in these cells. This enzyme, which is developed during the germination of the seed, can be extracted from the malt by cold water, and precipitated from this solution by alcohol. As another example of the fermentative decomposition of cellulose may be quoted the formation of marsh gas according to the equation

$$C_6H_{10}O_5 + H_2O = 3CO_2 + 3CH_4$$

which may be observed when vegetable matter is undergoing slow decomposition under stagnant water.

### CHARACTERS AND PROPERTIES OF COMPOUND CELLULOSES.

As already stated, the main characteristic of the group of compound celluloses is that they are composed of one or other form of cellulose combined with some other substances of a non-cellulose nature.

The nature of the *cellulose constituent* varies according to the source from which it is obtained, one of the chief characteristic differences between such different forms of cellulose being their behaviour on boiling with hydrochloric acid; thus whereas cotton cellulose yields only about 0·1-0·4 per cent of furfurol, jute cellulose under similar conditions yields 3·0-6·0 per cent, and straw cellulose yields from 12·0-15·0 per cent; for this reason the cellulose constituent is regarded as being of the nature of oxycellulose.

*The Non-cellulose Constituent* of compound celluloses may vary very considerably in chemical nature, and on this fact depends their classification into—

(*a*) Lignocelluloses.
(*b*) Pectocelluloses.
(*c*) Adipo- or Cuto-celluloses.

(*a*) *Lignocelluloses.*—In the young cell the walls consist of almost pure cellulose, but, as the cell grows older, the walls may become permeated with what are known as encrusting substances, the process being known as lignification. This change takes place at the expense of the cellulose, and new substances such as lignocellulose are produced. The extreme limit of this change is the production of wood, which contains only about 50-60 per cent of cellulose, while lignocelluloses still contain about 70-80 per cent.

The lignocelluloses are considered by most authors to consist of cellulose combined with at least two other non-cellulose constituents; one of these, A, appears to contain an aromatic nucleus, and the other, B, contains a furfural-yielding complex, and is possibly a pentosane. The two constituents, A and B, are sometimes grouped together as a single substance under the name of lignin or lignone, which contains 50-60 per cent carbon, as compared with cellulose, which contains only 44 per cent.

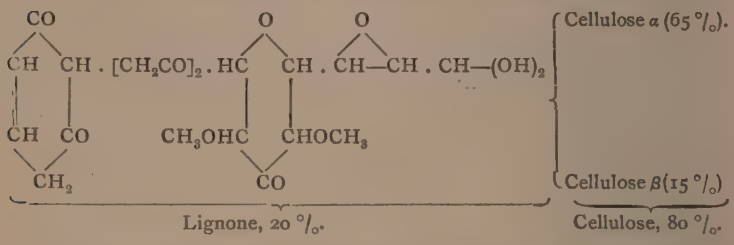
According to Klason,* spruce wood consists approximately of 50 per cent cellulose, 16 per cent other carbohydrates or lignosans, 30 per cent lignin, and 4 per cent other substances. The lignin would appear to be a condensation product of one molecule of coniferyl alcohol (p. 187), with three molecules of hydroxy coniferyl alcohol, having the formula $C_{40}H_{42}O_{12}$; the union between these compounds is effected by the elimination of the elements of water between the hydroxyl of an allyl alcohol group and the phenolic hydroxyl of another molecule.†

Dorée and Cunningham,‡ on the other hand, are opposed to this view of the constitution of lignin, on the ground that no vanillin results from the action of ozone on lignocellulose, whereas this substance is produced readily by the oxidation of coniferyl alcohol.

According to Cross and Bevan ("Researches," III., p. 104), the lignone complex is composed of a ketohexene group joined by —$CH_2CO$— groups to a pyrone ring with two methoxyl

* Klason: "Arkiv. Kem. Min. Geol.," 1908, **3**, No. 5, 1, and 1917, **6**, No. 15, 1.
† See also Hägglund: *id.*, 1918, **7**, No. 8, 1.
‡ Dorée and Cunningham: "J. Chem. Soc.," 1913, **103**, 677.

groups. Lignocellulose, as exemplified by jute fibre, is considered by these authors to be an ester-like compound of 20 per cent lignone (which yields no furfural) with 80 per cent cellulose consisting of 65 per cent of a resistant $\alpha$-cellulose and 15 per cent of a less resistant furfural yielding $\beta$-cellulose, which also contains $-OCH_3$ groups, as represented below :—




This formula receives support from the work of Dorée and Cunningham,* who find that ozonized oxygen acting on beech wood in the presence of water produces acid substances presumably by attacking the double bond of the lignone complex, and producing two carboxyl groups ; the pyrone ring containing the methoxyl groups is broken up yielding lower acids, amongst them acetic acid, probably produced from the $CH_2CO$ groups connecting the pyrone ring to the aromatic nucleus.

The lignocellulose type, according to Cross and Bevan (Lectures delivered before the Royal Society of Arts, Feb.-March, 1920), is not confined to jute and woody fibre, since the proportion of lignone complex in esparto grass is relatively high, but it is here not associated with the cellulose fibre. Esparto may be described as a structure of cellulose fibres embedded in a readily hydrolysable ligno-pentosan or ester-like compound of lignone with pentose.

It may here be remarked that although the best quality paper is manufactured from cellulose freed as completely as possible from non-cellulose constituents by the method described below, lignocelluloses are used as such for the preparation of inferior qualities of paper, without previous treatment for the removal of the lignin. Papers prepared from this mechanical wood pulp (see p. 161) when dipped in a 1½ per cent. alcohol solution of phloroglucinol and touched with a drop of diluted

* Dorée and Cunningham : "J. Chem. Soc.," 1913, 103, 677.

hydrochloric acid are coloured red. They are, moreover, turned yellow by exposure to sunlight.

(*b*) *Pectocelluloses.*—The most important example of a pectocellulose is flax; pectocelluloses occur also in ramie fibre, hemp, and raw cotton. Their outstanding characteristic is that their non-cellulose constituent is a pectic substance; so that on boiling with dilute alkali they give a product which gelatinizes in cooling, and from which, on the addition of acid, a precipitate of pectic acid (see p. 146) may be obtained. The pectocelluloses are possibly not true compounds, but merely associated deposits of celluloses with hemicelluloses or pectic substances.

The widely distributed *mucocelluloses* comprise such substances as quince or salep mucilage; both these substances while containing a certain amount of inorganic material give on analysis formulæ approximating to $C_6H_{10}O_5$. On hydrolysis with dilute acid they yield varying amounts of insoluble cellulose, together with soluble gums and monosaccharides, such as glucose or even a pentose.

Schwalbe is of opinion that Cross and Bevan's classification is not justified, and considers that these compounds should not be grouped with cellulose at all, but should be regarded as mucilages (see p. 143).

(*c*) *Adipo- and Cuto-celluloses.*—Under this heading are included the constituents of suberized and cuticularized walls. These substances are by some regarded as ester-likecompounds* of cellulose, with fat or wax-like materials, known as suberin and cutin. This view is based upon the fact that suberized walls, if treated first with a solution of potash, turn with chlorzinc iodide a red-violet colour.

The work of Gilson,† however, tends to show that cellulose does not enter into the composition of such walls, for the following reasons:—

1. Cellulose is not attacked by prolonged boiling in a 3 per cent solution of potassium hydrate in alcohol; suberized walls, on the other hand, are dissolved.

2. Phellonic acid ($C_{22}H_{43}O_3$) has been isolated from cork,

* Cross and Bevan: "J. Soc. Dyers and Colourists," 1919, 35, 70.
† Gilson; "La Cellule," 1890, 6, 63.

and this substance, together with its potassium salt, gives a red coloration with chlorzinc iodide. This suggests that the coloration of suberized membranes with chlorzinc iodide after treatment with potash is due to the presence of potassium phellonate and not to cellulose, for, in addition, the coloration does not take place if the corky tissue be subjected to the action of boiling alcohol after treatment with potash.

3. After treatment with cuprammonia, the chlorzinc iodide gives a yellowish-brown colour: this, according to Gilson, is due to the alteration of potassium phellonate into the copper salt, and not to the removal of cellulose, as had been supposed.

Gilson separated from oak-cork suberic acid ($C_{17}H_{30}O_3$) and phloionic acid ($C_{11}H_{21}O_4$) in addition to phellonic acid. He does not think that these occur as true glycerine esters, since the suberin walls are insoluble in all fat-solvents, and do not melt at a temperature below 290° C.

These observations have been supported by van Wisselingh,* who finds that cork does not contain cellulose, and that the suberin constituents are mostly soluble in chloroform, and melt at a temperature below 100° C. He concludes that suberin consists of fatty substances with glycerol or other compound esters easily decomposed by potash.

Gilson's views also find support in the observations of Schmidt,† who concludes that suberin and cutin are mixtures of polymerized fatty acids and glycerine-esters. The matter, however, cannot be considered as finally settled.

## CONSTITUTION OF CELLULOSE.

The following characteristics of this substance throw some light on the constitution of cellulose:—

1. On hydrolysis it yields dextrose.‡

2. On partial hydrolysis it sets free —CO groups which are present in some suppressed form in ordinary unchanged cellulose.

* van Wisselingh: "Chem. Centr.," 1892, ii., 516.
† Schmidt: "Monatshefte," 1910, 31, 347.
‡ See Cunningham: "J. Chem Soc.," 1918, 113, 173.

3. The fact that on destructive distillation it yields acetic acid and methyl alcohol points to the presence in the molecule of a $—CH_2—CO—$ grouping.

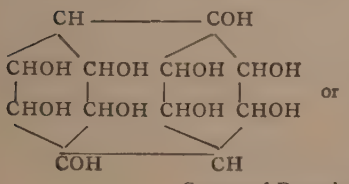
4. It is a very stable substance, which resists alkalis, oxidizing agents, and, to some extent, acetylation, except in the presence of a condensing agent, such as zinc choride or sulphuric acid.

5. With strong acids it yields esters, e.g. nitrates, acetates, and benzoates.

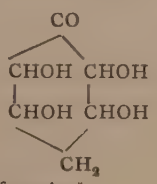
6. It undergoes the thio-carbonate reaction by treating alkali cellulose with carbon disulphide, when a change, which may be represented by the following equation, takes place :—

$$RONa + CS_2 = CS\begin{matrix}OR\\SNa\end{matrix}$$

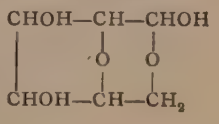
Various alternative structural formulæ have been suggested.



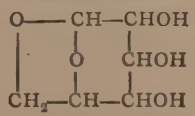

or




Cross and Bevan's formulæ.*




Green's formula.†




Vignon's formula.‡

Green § is of opinion that although cellulose is a colloid it does not follow that it has a high molecular weight. He considers that his formula well explains the following facts among others :—

1. That the highest nitrate obtainable from a cellulose molecule containing six carbon atoms is a trinitrate.‖

* Cross and Bevan : "J. Chem. Soc.," 1901, 79, 366.

† Green and Perkin : *id.*, 1906, **81**, 811.

‡ Vignon : "Bull. Soc. Chim.," 1899, **21**, 599.

§ Green : "Zeit. f. Farb. u. Textil Chemie," 1904, **3**, 97, 309.

‖ It should be clearly understood that by the nitration of cellulose it is possible to obtain a whole series of esters, representing different degrees of nitration. These various compounds may be described as mono-, di-, tri-, etc., up to deka-, or possibly dodeka-nitrates of a cellulose molecule containing twenty-four

2. That the highest acetate obtainable from a cellulose molecule containing six carbon atoms is a tri-acetate.

3. That cellulose does not react with phenylhydrazine, but on hydrolysis readily yields carbonyl groups which are able to react. The formula and its arguments are, however, not accepted by Cross and Bevan.*

Dreaper regards cellulose as a typical colloid, and, as a consequence, considers that it has no reacting unit such as a crystalline body has, nor has it a fixed molecular constitution such as can be represented by any constitutional formula; its reacting unit at any moment is a function of the condition under which it is placed.

### INDUSTRIAL USES OF CELLULOSE AND CELLULOSE PRODUCTS.

One of the industries which consumes the largest amount of cellulose is that of paper manufacture. Formerly the chief sources of cellulose for this purpose were cotton or hemp fibres; but with the increased consumption of paper other sources had to be found. Although straw contains cellulose which has been only slightly lignified, it is found to be unsuitable for the preparation of pure cellulose, owing to the fact that it contains a considerable quantity of silica. The employment of wood as a source of cellulose became possible with the discovery of chemical methods of destroying the non-cellulose constituent lignin, i.e. the "encrusting substances," without affecting the cellulose proper.

In the manufacture of paper from linen rags or cotton waste the material is cut up, cleaned, and disintegrated by boiling successively with dilute sodium carbonate and caustic soda under pressure; the fibre is then bleached with chlorine, the excess being subsequently removed; it is then treated with resin, soap, and alum, and spread in thin layers and dried, whereby the fibres become felted together in a peculiar manner, with the formation of paper. When wood is used the "encrusting substances" may be removed by boiling with calcium bisulphite, whereby the lignin remains in solution and a fairly pure form of cellulose, known as sulphite cellulose, is

carbon atoms. What is commonly called cellulose hexanitrate, the substance employed in the manufacture of gun-cotton is calculated on a $C_{12}$ molecule, which, therefore, corresponds to a trinitrate of a $C_6$ molecule.

* Cross and Bevan: "Zeit. f. Farb. u. Textil Chemie," 1904, 3, 197.

produced. In the preparation of inferior quality papers there is no chemical treatment of the disintegrated wood pulp; the material is, therefore, known as mechanical pulp, and paper made from it gives reactions for lignocellulose. Cellulose used for the preparation of filter papers is, after the ordinary methods of purification, treated with hydrofluoric acid to remove silica.

### COMMERCIALLY VALUABLE DERIVATIVES OF CELLULOSE.

When heated in a concentrated solution of zinc chloride, cellulose is converted into a viscid syrup. This syrup, when forced through glass nozzles into alcohol, forms threads which, after being washed and carbonized, become hard and are used for electric lamp filaments; they have also been employed for the basis of incandescent lamp mantles.

*Gun Cotton or Pyroxylin.*—That a variety of different products may be obtained by the action of various strengths of nitric acid, either alone or in the presence of sulphuric acid, on cellulose, has already been mentioned. The substance known as gun cotton is a hexanitrate; it is obtained by immersing dry cotton waste, freed from grease by treatment with alkali, in a mixture of 1 part nitric acid (sp. gr. 1·52) with 3 parts sulphuric acid (sp. gr. 1·84); the resulting substance is then rapidly and thoroughly washed with water, moulded into discs, and dried on heated plates. On explosion it produces corrosive gases and therefore is not suitable for use, as such, in firearms; when, however, the gun cotton is dissolved in ethyl acetate or acetone and the solution is evaporated, a new substance is obtained which has the same composition as gun cotton, but different properties; it explodes with less violence and produces no corrosive vapours, and is therefore employed in the manufacture of smokeless powder.

*Blasting Gelatine* is a mixture of gun cotton and nitroglycerine. Gun cotton mixed with a variety of other substances enters into the composition of numerous explosives, such as ballastite, melanite, cordite, etc. etc.

*Collodion* is the name applied to a solution of cellulose tri- and tetra-nitrates in a mixture of equal parts of 95 per cent alcohol and ether.

A substance known as *artificial india-rubber** is produced by kneading together a mixture of tri- and tetra-nitrocelluloses partially dissolved in ether alcohol with castor oil. The resulting substance may be made to have any degree of elasticity, according to the materials which are mixed with it. It forms a more or less satisfactory substitute for rubber and possesses a high electric resistance. Though not explosive, it is inflammable, but to do away with this inconvenience the outer surface may be denitrated by treatment with alkali, whereby it is rendered non-flammable. *Artificial gutta-percha* is obtained by allowing an acetone solution of tetra-acetyl cellulose to evaporate.

*Celluloid* is produced by mixing the tri- and tetra-nitrates, as employed for collodion, with camphor.

*Artificial Silks.*—These are produced in a variety of ways by precipitating some form of cellulose from solution. The first artificial silk was prepared by Chardonnet, who obtained it by forcing collodion through fine nozzles; the thin stream of nitrocellulose solution on coming in contact with the air solidifies to a thread by the rapid evaporation of the solvent. To render it non-flammable the thread is denitrated by treatment with ammonium sulphide.

A second process for preparing artificial silk consists in dissolving bleached mercerized cotton (see p. 151) in cuprammonia solution. A fine stream of this solution is then run into a dilute sulphuric acid, whereby a continuous thread of cellulose is at once precipitated.

A third process is that in which viscose solution is forced through fine nozzles, the emerging streams being coagulated either by hot air or by a bath of ammonium chloride. The fine threads which result can be spun like silk. Cellulose acetate also is used for this purpose.

*Viscose* is obtained by acting on finely divided cellulose with soda and treating the resulting substances with carbon disulphide, whereby a cellulose thio-carbonate is produced; this substance on exposure to air decomposes spontaneously

* This substance must be carefully distinguished from so-called synthetic rubber, which is an artificially polymerized hydrocarbon of the formula $(C_5H_8)^n$; this substance, if not actually identical with natural rubber, is at any rate closely related to it, whereas the artificial india-rubber mentioned above is a nitrated cellulose.

into cellulose alkali and carbon disulphide. Viscose solutions are employed for sizing paper and in the manufacture of wall-papers.

Mixed with metallic dust and colouring matters, viscose can be converted into an artificial leather, and may also be employed for rendering canvas waterproof and for making cinematograph films, etc.

*Viscoid*, which is congealed viscose, is a hard mass obtained by mixing viscose with various substances and allowing the mixture to decompose spontaneously and harden; it is used for mouldings, cornices, statuettes, etc.*

*Solid Spirit.*—The substance sold under this name is obtained by pouring a solution of cellulose acetate in glacial acetic acid into alcohol; a white solid is produced which does not melt, and burns when ignited without leaving any ash.

Cellulose acetate, in which there are approximately five acetyl groups to the $C_{12}$ cellulose unit, is soluble in acetone, and is used largely as a dressing for the fabric of aeroplane wings.

*Cellite* is acetyl cellulose which is soluble in a mixture of ethyl acetate and ethyl alcohol. Mixed with camphor it is used in the manufacture of non-flammable cinematograph films.

*Willesden paper* is paper waterproofed by treatment with cuprammonia, whereby the fibres are gelatinized, and, when dry, are impervious to water.

Finally, mention may be made of a few substances which are made from cellulose as a starting point, but which are produced only by the profound decomposition of the molecule. Thus by heating cellulose with a strong solution of caustic potash and soda, oxalic acid is produced, and by the destructive distillation of wood, acetic acid, acetone and methyl alcohol are obtained.

### MICROCHEMICAL REACTIONS.

#### A. *Normal Cellulose.*

1. With a dilute solution of iodine a yellow coloration results.

* See Bersch: "Cellulose, Cellulose Products and Artificial Rubber," Philadelphia, 1904.

2. After staining well with iodine, the addition of strong sulphuric acid causes the cellulose walls to swell considerably and to turn blue.

3. Chlorzinc iodide causes swelling, accompanied by the assumption of a blue colour.

4. Calcium chloride iodine solution turns pure cellulose dull pink to violet without swelling.

Zimmermann gives the following directions for making this reagent. A concentrated solution of calcium chloride is made, and for each 10 c.c. of this solution there is added ·5 gram of potassium iodide and ·1 gram of iodine. The mixture is then gently heated and filtered through glass-wool.

5. Pure cellulose is easily soluble in cuprammonia.

6. The hemi-celluloses give different reactions; some turn blue with dilute iodine, and either do not dissolve in cuprammonia, or only after prolonged treatment.

### B. *Compound Celluloses.*

#### (*a*) *Lignin.*

1. A brownish-yellow colour is given with iodine.

2. The addition of strong sulphuric acid, after previous treatment with iodine, turns lignified walls brown.

3. The same colour is obtained with the use of chlorzinc iodide.

4. Calcium chloride iodine solution turns lignin yellow to yellow-brown.

5. Insoluble in cuprammonia.

6. Aniline sulphate or aniline chloride in aqueous solution and acidified with the appropriate acid turns lignified walls a bright yellow.

7. If the sections be soaked for about a minute in an alcoholic solution of phloroglucin (or resorcin, hydroquinone, pyrogallol, or pyrrole) and then mounted in a drop of strong hydrochloric acid, the lignified walls are turned a bright red.

8. A concentrated solution of thallin sulphate in 50 per cent alcohol gives a yellow to orange-yellow coloration.

The sections should be treated first with alcohol, and the thallin sulphate solution should be freshly prepared.

The colour-reactions obtained by the use of aniline sul-

phate, thallin sulphate, phloroglucin and the other reagents mentioned in paragraph 7, are due to the presence of a furfural-yielding complex in the lignin; any substance in the plant which contains this complex, e.g. coniferin, will give similar reactions.

9. If lignified tissues be treated with chlorine water and then with sodium sulphide, a deep magenta colour is produced.

10. Lignocelluloses induce the formation of Prussian blue in the greenish-red solution produced by mixing ferric chloride with potassium ferricyanide.

### (*b*) *Suberin and Cutin.*

1. With chlorzinc iodide, and also with iodine and sulphuric acid, a brown or yellow colour is given.

2. Suberized and cuticularized walls are insoluble in cuprammonia and concentrated sulphuric acid.

3. Suberized walls are coloured yellow with strong potash solution; on heating the colour deepens, and on boiling yellow oily drops exude from the membranes.

4. Suberized walls are the most resistant of membranes to Schultze's macerating mixture; but on boiling, oily drops of ceric acid are formed which are insoluble in carbon bisulphide but soluble in ether, benzol, and hot alcohol.

5. Suberized and cuticularized walls are stained green by the action of alcoholic solutions of chlorophyll. A strong fresh solution of chlorophyll must be used, and the treatment should last for at least fifteen minutes in the dark. The sections may be washed in and examined in water. (Lignified walls are unacted upon by this and the following reagents.)

6. Similarly the same membranes are stained red by treatment with alcoholic solutions of Alkannin, Sudan III and Scharlach R.

7. If a section of the material be treated first with eau de Javelle, in order to destroy any tannins which may be present, suberized walls are stained very deeply with a solution of cyanin in 50 per cent alcohol to which an equal volume of glycerin has been added. Lignified walls will not be stained owing to the preliminary treatment with the eau de Javelle.

8. Corky walls are stained orange-yellow by an alcoholic

solution of *extractum orleanæ* spirit which must be filtered before using.

FURTHER REFERENCES.

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# SECTION IV.

## GLUCOSIDES.

THE glucosides are compounds of some complexity which on decomposition yield glucose together with one or more other substances, usually of an aromatic nature; they are, therefore, often described as ether-like compounds of carbohydrates with aromatic compounds. The carbohydrate in the majority of cases is glucose, but occasionally it may be an isomeric hexose, such as galactose or even a pentose such as rhamnose. Thus, for example, digitonin, one of the glucosides of *Digitalis purpurea*, yields on hydrolysis both glucose and galactose, while hesperidin and quercitin, the glucosides contained respectively in the unripe orange and in the bark of *Quercus tinctoria*, give rhamnose.

The reaction by means of which glucosides are split up into their constituent parts is in almost all cases one of hydrolysis,* and can, therefore, usually be brought about by boiling with dilute mineral acids or, in some cases, alkalis. In nature, however, the decomposition is effected by means of suitable ferments which often exist in the same part, although generally in different cells to the glucoside.

Each glucoside may have an enzyme appropriate to itself, but any one particular ferment may have the power of splitting several glucosides.

Thus, for example, the glucoside amygdalin is hydrolysed by its appropriate enzyme emulsin to glucose, benzaldehyde and hydrocyanic acid, according to the equation :—

$$C_{20}H_{27}NO_{11} + 2H_2O = 2C_6H_{12}O_6 + C_6H_5CHO + HCN$$

* The decomposition of potassium myronate into potassium hydrogen sulphate, glucose and allyl mustard oil can hardly be described as hydrolysis (see p. 186).

whilst the enzyme amygdalase,* contained in yeast, is only able to effect a partial hydrolysis to glucose and mandelonitrile glucoside.†

$$C_{20}H_{27}NO_{11} + H_2O = C_6H_{12}O_6 + C_{14}H_{17}NO_6$$

On the other hand, emulsin can hydrolyse other glucosides, besides amygdalin, such as salicin, arbutin, etc.

### THE CONSTITUTION OF THE GLUCOSIDES.

The constitution of the natural glucosides can be best understood by a brief consideration of the simplest known artificial glucosides which have been synthesized from glucose.

The $\gamma$ lactone formula for glucose (see p. 67) with its asymmetric terminal carbon atom accounts for the ability of glucose to react with methyl alcohol to form two isomeric $\alpha$- and $\beta$-methyl glucosides ‡ according to the equation :—

$$CH_2OH\,CHOH\,\overset{\overset{\_\_\_\_O\_\_\_\_}{\vert\qquad\qquad\vert}}{CH\,CHOH\,CHOH\,CH}OH + CH_3OH$$

$$= CH_2OH\,CHOH\,\overset{\overset{\_\_\_\_O\_\_\_\_}{\vert\qquad\qquad\vert}}{CH\,CHOH\,CHOH\,CH}OCH_3 + H_2O$$

The $\alpha$-glucoside, which is dextro-rotatory, is hydrolysed by maltase, but not by emulsin, while the $\beta$-glucoside, on the contrary, is unaffected by maltase, but is hydrolysed by emulsin. §

As the result of studying the action of these two enzymes upon other glucosides, Fischer divides these substances into two classes known as $\alpha$-glucosides and $\beta$-glucosides, according as they are hydrolysed by maltase or emulsin respectively. Amygdalin is, therefore, an $\alpha$- $\beta$-glucoside, since of its two glucose molecules, one is the $\alpha$- and the other is the $\beta$-modification.

Since most natural glucosides yield on hydrolysis a substance containing a hydroxyl group, it seems reasonable to

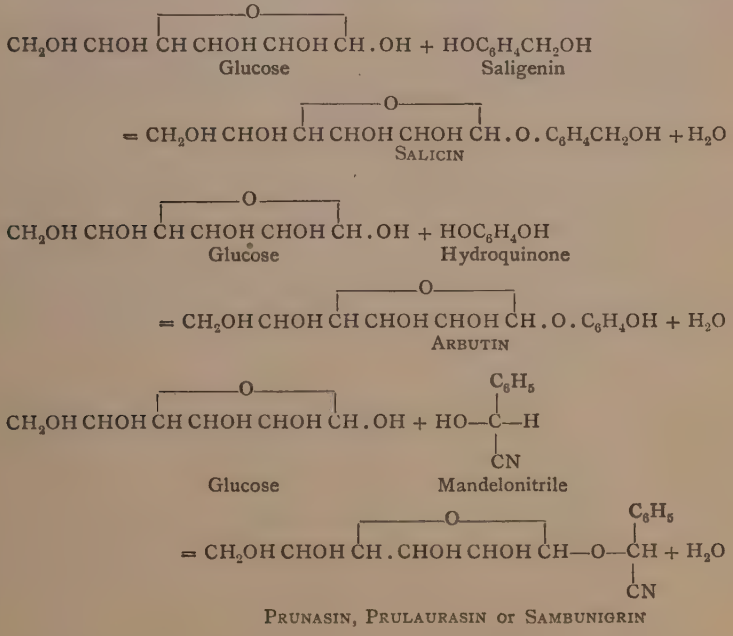
* Caldwell and Courtauld: "Proc. Roy. Soc., Lond.," B., 1907, **79**, 350.

† Fischer: "Ber. deut. chem. Gesells.," 1899, **28**, 1509.

‡ A number of analogous compounds have since been prepared by Fischer and his co-workers from mannose, galactose, and fructose, the resulting compounds being termed mannosides, galactosides, and fructosides respectively.

§ See also section on Enzymes, p. 355.

assume that the original glucoside was formed by a reaction similar to the one given for the artificial glucosides mentioned above, that is by the elimination of water between a hydroxyl group of the sugar and one from the other compound. On this assumption the constitution of some of the better known natural glucosides could be represented as follows :—


CH2OH CHOH CH CHOH CHOH CH . OH + HOC6H4CH2OH
Glucose
Saligenin
= CH2OH CHOH CH CHOH CHOH CH . O . C6H4CH2OH + H2O
Salicin
CH2OH CHOH CH CHOH CHOH CH . OH + HOC6H4OH
Glucose
Hydroquinone
= CH2OH CHOH CH CHOH CHOH CH . O . C6H4OH + H2O
Arbutin
C6H5
CH2OH CHOH CH CHOH CHOH CH . OH + HO—C—H
CN
Glucose
Mandelonitrile
C6H5
= CH2OH CHOH CH . CHOH CHOH CH—O—CH + H2O
CN
Prunasin, Prulaurasin or Sambunigrin

In some cases the natural glucosides have been actually synthesized; thus salicin has been obtained by the reduction of the corresponding aldehyde glucoside, helicin :—

$$C_6H_{11}O_5 . O . C_6H_4CHO + 2H = C_6H_{11}O_5 . O . C_6H_4CH_2OH$$

the helicin itself having been synthesized from glucose and salicylic aldehyde.

### *Identification.*

For the identification of glucosides the character of the cleavage products are relied upon; these products, with the

exception of sugar, are frequently of a volatile nature and possess a characteristic smell; their chemical nature, however, varies so much that there is no general test for the whole group other than that afforded by the presence of a carbohydrate after hydrolysis.

The method devised by Bourquelot has been much employed; the expressed sap of the plant is examined as to its power of reducing Fehling's solution and as to its optical properties, the ferment emulsin is then added to the extract and the mixture kept in the warm for a time. The amount of sugar is thereupon again estimated and so also is its rotatory power. The increase indicates roughly the amount of glucoside originally present.

In microchemical work the same test, in a simplified form, may be applied, the preparation being treated with a solution of emulsin or of dilute sulphuric acid, and then gently warmed with Fehling's solution.

These tests, however, are of value only in certain cases, for some glucosides, and for that matter some other substances likewise, can reduce Fehling's solution without undergoing a preliminary hydrolysis, also there may be present in the cells of the plant other substances which on hydrolysis would yield glucose, and further some glucosides like gynocardin exhibit a remarkable resistance to the action of acids.

The decomposition products of many glucosides are brightly coloured, for example, rhinanthin when boiled with dilute hydrochloric acid yields a dark blue-green colour which is due to rhinanthogenin. Several give a brilliant red coloration with strong sulphuric acid, e.g. salicin and phloridzin. Most glucosides are soluble in varying degrees in either hot or cold water and alcohol; the majority are insoluble, or nearly so, in ether, which fact is made use of in separating them from alcoholic solutions.

### PHYSIOLOGICAL SIGNIFICANCE OF GLUCOSIDES.

In attempting to assign the part played by these substances in the economy of the plant, it must be remembered that the number of glucosides of natural occurrence are very numerous and, in some cases, of a diverse nature; it is, therefore, possible that the significance of the presence of one glu-

coside may be quite different to that of another, but even in the case of glucosides of the same nature there is much diversity of opinion. They have been described, on insufficient grounds, as direct products of photosynthesis. Many consider them to be of value as food-stuffs on account of the sugar they contain; the occurrence of certain glucosides in seeds lends some support to this view, for in the case of the bitter almond hydrocyanic acid, in the free state, may be identified when germination starts, also the observations of Treub,* who found that in the case of some plants containing cyanogenetic glucosides the amount of the latter decreased if the plant was placed in the dark, in order that photosynthesis could not take place. On the other hand there was an increase in quantity when the plants were exposed to light, and this increase reached a maximum at about midday.

Weevers† considers that salicin, populin, arbutin and similar glucosides are of the nature of reserve food-materials, for not only is the formation of these substances a suitable means for the storage of sugar on account of their low diffusibility, but the facts of their seasonal or diurnal variation lend support to this opinion. Thus in *Vaccinium Vitis-Idæa* the arbutin is stored in the leaves, and when the new leaves are formed in the spring it is used up; it is split by a suitable enzyme, the sugar is used up, and the hydroquinone remains behind and combines with more sugar, so that by the autumn the leaves once more contain much arbutin.

In the case of the willow, salicin is formed day by day, but during the night it is split by salicase into sugar and the alcohol saligenin. The glucose is translocated, and the saligenin remains behind and is converted into salicin by combining with sugar the next day. This process stops in the autumn, by which time there is relatively much salicin in the cortex of the stem.

This translocation of glucosides from the leaves of many plants—but not of all, *Sambucus* and *Indigofera* being exceptions—is significant, and so also are the facts relating to the

* Treub: "Ann. Jard. Bot. Buitenzorg," 1896, **13**, 1; 1907, **21**, 79, 107; 1910, **23**, 85.

† Weevers: "Kon. Akad. Wet.," Amsterdam, 1902; "Rec. Trav. Bot. Néerl," 1910, **7**, 1.

amount of glucosides in the bark and other parts of plants at different seasons of the year. Thus in *Salix* and *Populus* the glucoside (salicin) is most abundant in the autumn and winter, and is used up in the following spring during the period of flowering and seed formation; also in the case of *Taxus* the glucoside (taxicatin), which appears principally in the young shoots, is greatest in amount in the autumn and winter. In *Pangium edule* and other plants the amount of cyanogenetic glucosides is greatest in young leaves, with increasing age the amount diminishes.

Guignard * does not believe that glucosides, or at any rate the cyanogenetic ones, are reserve food stuffs, since, if introduced into the food materials of a plant, glucosides have an injurious effect, owing to the aromatic residues.

Combes,† however, finds that a glucoside is toxic only to plants in which it does not naturally occur; he agrees that glucosides do not furnish carbohydrate food, since plants grown in an atmosphere free from carbon dioxide are unable to make use of these substances.

Peche‡ holds that hydrocyanic acid is a direct product of photosynthesis; some of it combines with sugar to form a glucoside, and some is transported in a labile form, probably in a loose combination with tannin, and stored for future use as food in various tissues.

The occurrence of certain glucosides, especially in places of active metabolism such as leaves and young shoots, may indicate that certain bye-products are fixed, either temporarily or more permanently, in this form.

In conclusion it may be stated that many may perform a biological function; thus the bitterness or poisonous nature of the glucosides or of the products of hydrolysis, other than sugar, may serve as a protection against herbivorous or fruit-eating animals; the antiseptic properties of these dissociation products may have a value in preventing the development of disease organisms in parts which may be damaged, e.g. seeds, leaves and bark. Some may play a part in connexion with

* Guignard: "Compt. rend.," 1905, **141**, 236; 1906, **143**, 451.
† Combes: "Rev. gen. Bot.," 1918, **30**, 216.
‡ Peche: "Sitz. Kais. Akad., Vienna," 1912, **121**, 33.

the secretion of sugar by extrafloral nectaries, for it appears that the basal cells of these structures, together with the elements of the adjacent tissues, are rich in glucosides.

## CYANOGENETIC GLUCOSIDES.

Among the more important glucosides are the cyanogenetic ones, so named because on hydrolysis they yield hydrocyanic acid as one of the products.

Hydrocyanic acid is of fairly common occurrence in the higher plants, and although sometimes it occurs in the free state it is, in the majority of cases, combined; the nature of many of these compounds has not yet been ascertained, but it is not improbable that generally they are glucosides.

Cyanogenetic glucosides, although widely distributed, are somewhat rare when compared with other glucosides such as the saponins. Hydrocyanic acid has been found in a few Fungi, and in certain plants of the following Natural Orders of the higher plants: Polypodiaceæ, Aroideæ, Gramineæ, Sapindaceæ, Sapotaceæ, Proteaceæ, Ranunculaceæ, Papaveraceæ, Magnoliaceæ, Lauraceæ, Droseraceæ, Rosaceæ, Saxifragaceæ, Leguminosæ, Platanaceæ, Euphorbiaceæ, Compositæ, etc. It will be observed from this list that some Cohorts, for example Rosales and Ranales, stand out in having several natural orders characterized by the presence of the substance in question.

In the individual plant the cyanogenetic glucosides occur more especially in the leaves and buds, in the seed, and also in the bark.

In *Pangium edule* Treub* found such glucosides in the phloem, pericycle, and in special cells of the leaves; Guignard† describes such compounds as occurring in the leaves of vigorous shoots, the young bark, and in the unripe fruit of *Sambucus nigra* and species of *Ribes*. The amount present in a member is not constant; Verschaffelt‡ found that as the buds of *Prunus Padus* and *P. Laurocerasus* open, the amount of hydrocyanic compounds increases as rapidly as do the other

* Treub: "Ann. Jard. Bot. Buitenzorg," 1907, 21, 107.
† *Loc. cit.*
‡ Verschaffelt: "Kon. Akad. Weten. Amsterdam," 1902.

substances present. Treub has found that in plants growing in the tropics and which contain cyanogenetic glucosides, these substances disappear before leaf-fall; in some cases this depletion is quite sudden, in others the glucosides gradually disappear. On the other hand in *Indigofera* and *Sambucus* the glucosides are not removed before the fall of the leaves.

Treub also states that the amount present depends on the quantity of available sugar; he observed that there obtains a daily variation, the maximum quantity occurring at about midday. On the other hand, there is no consistent daily fluctuation in *Sorghum*, and unhealthy plants may contain more than healthy. It has also been ascertained that the quantity of cyanogenetic glucosides in *Pangium*, *Phaseolus lunatus*, *Zea* and *Sorghum* may be increased by the application of manures rich in nitrates; on the other hand, it must be pointed out that in some cases, e.g. *Phaseolus lunatus*, the glucoside may be eliminated from the seed by suitable methods of cultivation. Also the amount varies in different varieties of species, e.g. *Sorghum*. In some examples of seeds which contain little or no hydrocyanic acid there may be a marked increase on germination, thus in the flax, Dunstan and Henry * found that the seeds contained ·008 per cent of the acid, whereas in the seedlings ·135 per cent obtained; the same increase also occurs in the sweet almond. Further, the percentage of hydrocyanic acid in *Linum*, *Sorghum*, *Lotus arabicus* and *Zea Mais* gradually increases to a maximum and then decreases, sometimes to zero.

The stage of development at which the maximum is reached varies in the different plants; thus, to take two extreme cases, in the flax the maximum obtains when the seedlings are between four and five inches high, whilst in *Lotus arabicus* the maximum occurs at the period of flowering.

From these observations it is clear that the actual amount of the substance in question varies pretty considerably; it may be very small or relatively large, thus in the young leaves of *Pangium* the presence of ·3 per cent of hydrocyanic acid has

* Dunstan and Henry: "Brit. Assoc. Rep., York," 1906; "Phil. Trans. Roy. Soc., Lond.," 1901, B., 194, 515; "Proc. Roy. Soc. Lond.," B., 1900, **67**, 224; 1901, **68**, 374; 1903, **72**, 285.

been ascertained. In this connection attention may be drawn to observations by Greshoff,* who states that the absence of hydrocyanic acid does not necessarily indicate the absence of a cyanogenetic glucoside. If the glucoside produced be very small in amount, as in the case of *Xeranthemum*, the hydrocyanic acid may be used up directly it is formed, so that benzaldehyde only will be found as a decomposition product.

### ISOLATION OF CYANOGENETIC GLUCOSIDES.

Dunstan and Henry give the following method for the isolation of dhurrin from *Sorghum vulgare*. The plants are dried at a low temperature and ground up as finely as possible. The material so obtained is extracted with alcohol and filtered; the alcohol is then distilled off from the filtrate and the residue dissolved as completely as possible in warm water. Lead acetate is added to this aqueous solution until no more precipitate (chiefly lead tannate) comes down. A current of sulphuretted hydrogen—a large excess is to be avoided—is then passed through the filtrate and the lead sulphide filtered off. The excess of sulphuretted hydrogen can be removed from the filtrate by passing through it a current of air. The liquid is then worked up with pure animal charcoal, sufficient in amount to convert the whole, when dry, into a powder, and dried in a vacuum desiccator. When quite dry the material is extracted with anhydrous ethyl acetate in a Soxhlet apparatus; this solvent slowly removes the glucoside, leaving most of the sugar and other impurities behind. On distilling off the solvent a syrup remains which may, if necessary, be again treated in the same fashion. The syrup will deposit crystals of the glucoside after standing for a few days in a vacuum over sulphuric acid. The crystals so obtained may be recrystallized from hot alcohol or boiling water.

### CHEMISTRY OF CYANOGENETIC GLUCOSIDES.

We may now pass on to a brief consideration of the chemical nature of the cyanogenetic glucosides. These glucosides vary in different plants. Thus comparing the cherry laurel, *Prunus Laurocerasus*, with *Pangium edule*, it has been found

*Greshoff: "British Assoc. Rep., York," 1906, 138; "Kew Bull.," 1909, 397.

that in the former plant the localization of the glucoside is not so clearly defined as in the latter; also this substance disappears from the leaves of the cherry laurel, when kept in the dark, much more slowly than does the glucoside in *Pangium* on similar treatment. This indicates that these two glucosides have a different chemical constitution, and analysis has shown this to be the case. In *Pangium edule*, and also in *Linum* and other plants, the glucoside has an acetone cyanhydrin residue, while in the case of *Prunus* the residue is benzaldehyde cyanhydrin. The former glucosides are less stable than the latter.

With regard to the stages which lead up to the formation of prussic acid and its compounds, Gautier has put forward the supposition that it may possibly be formed by the action of formaldehyde on nitrates, and this view is not inconsistent with the distribution of nitrates in the leaves of some plants, but nothing definite is known.

### *Reactions, Microchemical and Otherwise.*

1. The presence of cyanogenetic glucosides or of free hydrocyanic acid can generally be detected by chewing a small piece of the material.

2. Thoroughly crush the part it is desired to examine under water and set it aside for some time, then filter and add to the filtrate a little silver nitrate; a white precipitate indicates hydrocyanic acid, but this test must be used with caution as many other substances give a white precipitate with silver nitrate.

If the amount of enzyme present in the tissue be very small, the maceration must be allowed to proceed for some time, or emulsin may be added to hasten the decomposition.

3. Cut a thick section of the fresh tissue to be examined and place it in a 5 per cent alcoholic solution of potash for about a minute; transfer to a solution containing 2·5 per cent ferrous sulphate and 1 per cent ferric chloride and keep at about 60° C. for ten minutes. Place the preparation in a dilute solution of hydrochloric acid—one part of strong acid to six parts of water—for five to fifteen minutes. The presence of hydrocyanic acid is indicated by the formation of Prussian blue.

If leaves are to be tested, instead of cutting them up they may be pricked all over with a bunch of fine needles and then treated as above.

4. Guignard's Test.—Dip strips of white filter-paper in a 1 per cent solution of picric acid and dry, moisten the papers again with a 10 per cent solution of sodium carbonate and again dry. The test papers, which may be kept in stoppered bottles for some time without deterioration, turn an orange red in the presence of fumes of hydrocyanic acid. The test is very delicate, and the rapidity of the change in colour depends on the amount of prussic acid present, so that if the quantity be very small the paper may have to be suspended in the test tube containing the material to be tested, for a day.

This test has been modified by Waller so as to give quantitative results, but it has been pointed out by Chapman* that the coloration is due to reduction, and is, therefore, not specific for hydrocyanic acid; accordingly the method must be used with caution.

It was found that if a leaf of the cherry laurel, *Prunus Laurocerasus*, be immersed in an aqueous solution containing ·05 per cent picric acid and 5 per cent sodium carbonate, the leaf is unharmed and the fluid undergoes no obvious change. If, however, the leaf be immersed in the same fluid to which chloroform has been added in the proportion of ·4 c.c. per 100 c.c. of fluid, the formation of hydrocyanic acid takes place and the sodium picrate turns red. The intensity of the colour is the basis of the quantitative estimation of the hydrocyanic acid.

The standard colour is obtained by mixing together equal volumes of the picrate fluid and ·002 per cent hydrocyanic acid. This mixture, which contains 10 mgs. of hydrocyanic acid per litre, is allowed to stand for twenty-four hours in an incubator kept at 40° C. The intensity of the colour is designated by the symbol T10, and corresponds to 10 mgs. of hydrocyanic acid per litre; a colour intensity of T1 similarly corresponds to 1 mg. of hydrocyanic acid per litre. By diluting the standard solution, a gamut of colour intensities may be obtained T1, T2, T3 . . . the figure in each case corresponding to the number of milligrams of hydrocyanic acid in one

* Chapman: "Analyst," 1910, 35, 469. See also Francis and Connell: "J. Amer. Chem. Soc.," 1913, 35, 1629.

litre of fluid. These colours may be matched closely by aqueous solutions of potassium bichromate.

In making up the standard solution it is important to allow the mixture to stand in an incubator kept at a temperature of 40° C. for certainly not less than an hour owing to the slowness of development of the full tint.

### AMYGDALIN.

Amygdalin, $C_{20}H_{27}NO_{11}$, is a lævo-rotatory bitter substance which is fairly soluble in water, and gives with concentrated sulphuric acid a pale reddish-violet coloration, this, however, is not a distinctive test, since the same coloration is given by other glucosides, e.g. menyanthin.

Amygdalin occurs in the seeds of the bitter almond, *Pyrus Amygdalus;* it is, however, generally stated not to occur in the seeds of the cultivated almond, the sweet variety, although emulsin, its appropriate enzyme, is present. Dunstan and Henry have shown that traces of hydrocyanic acid occur in the seeds, and more than traces in the seedlings, of the sweet almond; it is probable, therefore, that a small quantity of amygdalin does occur in the sweet variety. This relative absence of glucoside in the cultivated plant is important, and the same phenomenon has been found to obtain, by these same authors, in *Phaseolus lunatus.* The seeds of the wild plant yield large quantities of hydrocyanic acid, whereas those of the cultivated plants give very little or none.

Amygdalin has also been described as occurring in *Pyrus Malus*, *Pyrus Aucuparia*, *Pyrus cydonia* and other plants.

This glucoside is generally obtained by crushing the seeds of the bitter almond and subjecting the mass to considerable pressure between hot iron plates in order to remove the oil. The solid cake is then digested with hot alcohol which dissolves out the amygdalin. The alcoholic extract is evaporated down when the amygdalin separates out in scale-like crystals belonging to the monoclinic system.

It has already been mentioned that the appropriate enzyme generally occurs in the same tissues as the glucoside; this being so, the bitter almonds have only to be crushed in water in order to bring the ferment emulsin into contact with the amygdalin to bring about the hydrolysis.

This enzyme emulsin was originally supposed to be a single substance but it has since been found to be composed of two distinct enzymes known as amygdalase and prunase respectively. The former is only able to hydrolyse the amygdalin molecule into mandelonitrile glucoside (see p. 180) and glucose according to the equation :—

$$C_{20}H_{27}NO_{11} + H_2O = C_6H_{12}O_6 + C_{14}H_{17}NO_6$$

Mandelonitrile glucoside

while the prunase effects the further hydrolysis of the mandelonitrile glucoside :—

$$C_{14}H_{17}NO_6 + H_2O = C_6H_{12}O_6 + HCN + C_6H_5CHO$$

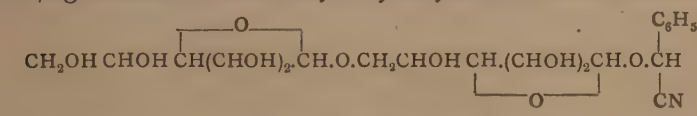
in the same way as it hydrolyses the natural glucoside prunasin of *Prunus padus* (for formula see p. 169), in which plant it occurs alone without amygdalase.

The two stages of the hydrolyses may be demonstrated either by carefully controlling the reaction of emulsin on amygdalin and stopping the reaction at the right moment, before the prunase is able to decompose the mandelonitrile glucoside, or else by hydrolysis by means of acids.*

An extract of yeast also only hydrolyses amygdalin as far as mandelonitrile glucoside; this was thought to be due to the action of maltase, but it has since been shown that this extract also contains amygdalase as distinct from maltase.

According to Giaja† amygdalin is broken up under the action of the gastric juice of a snail (*Helix pomatia*) into benzaldehyde, hydrocyanic acid and a disaccharide of unknown constitution.

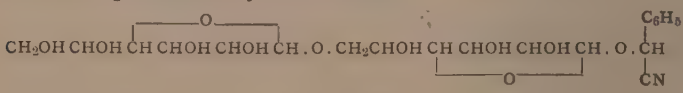
Although the constitution of amygdalin cannot be said to be established completely, it may be provisionally regarded as an $\alpha\beta$-glucoside of benzaldehyde cyanhydrin of the formula :—



If crystals of amygdalin be dissolved in water and then subjected to the action of maltase the hydrolysis will not proceed further than is represented in the first of the two

* Caldwell and Courtauld : "J. Chem. Soc.," 1907, 91, 666.
† Giaja : "Compt. rend.," 1910, 150, 793.

above equations; emulsin, on the other hand, can hydrolyse the mandelonitrile glucoside as indicated in the second equation, and, of course, it can bring about the whole series of changes. Since, moreover, the hydrolysis can be effected in two stages successively by the enzymes maltase and emulsin, which react with $\alpha$- and $\beta$-glucosides respectively, it follows that amygdalin must be an $\alpha\beta$-glucoside of benzaldehyde cyanhydrin as represented by the formula :—



The mandelonitrile glucoside * obtained by the partial hydrolysis of amygdalin is identical with the naturally occurring glucoside prunasin * contained in the twigs of *Prunus padus* and is isomeric with prulaurasin,* the glucoside occurring in the leaves of *Prunus laurocerasus*, and sambunigrin * which occurs in the fruit of the elder *Sambucus niger*. Sambunigrin has been synthesized by Fischer and Bergmann.†

The crude oil of bitter almonds contains hydrocyanic acid which may be removed by distillation with lime and ferrous chloride which converts the prussic acid into Prussian blue. Pure benzaldehyde is a colourless or pale yellow liquid, soluble in alcohol, but practically insoluble in water. Its specific gravity is 1·05, and its boiling point 180° C. On exposure to air it becomes converted into benzoic acid.

### DHURRIN.

This is a glucose closely allied to amygdalin, and occurs in the seedlings of *Sorghum vulgare*, but not in the older plants; it has the empirical formula $C_{14}H_{17}NO_7$ and yields, on hydrolysis, glucose, hydrocyanic acid and parahydroxybenzaldehyde :—

$$C_{14}H_{17}NO_7 + H_2O = C_6H_{12}O_6 + HCN + C_6H_4OHCHO$$

Similar glucosides occur in the seedlings of *Panicum* and *Zea*.

### PHASEOLUNATIN.

Phaseolunatin, $C_{10}H_{17}O_6N$, occurs in the seeds of wild

* For structural formulæ see p. 169.

† Fischer and Bergmann: "Ber. deut. chem. Gesells.," 1917, 50, 1047.

plants of *Phaseolus lunatus*, it is present only in very small quantities, or is entirely absent from the seeds of the cultivated plants. It is also present in *Linum* and many rubber-yielding plants, such as *Hevea braziliensis* and species of *Manihot*. Associated with it in its natural surroundings is the enzyme phaseolunatase which is able to hydrolyse it to acetone, glucose, and hydrocyanic acid,* from which it follows that phaseolunatin is a glucose ether of acetone cyanhydrin.

### LOTUSIN.

Lotusin, $C_{28}H_{31}NO_{16}$, occurs in *Lotus arabicus*. It is a bitter, yellow-coloured substance, which when fresh does not reduce Fehling's solution.

On hydrolysis it yields glucose, hydrocyanic acid, and lotoflavin, a bright yellow crystalline precipitate:—

$$C_{28}H_{31}NO_{16} + 2H_2O = 2C_6H_{12}O_6 + HCN + \underset{\text{Lotoflavin}}{C_{15}H_{10}O_6}$$

Lotusin, like dhurrin, does not occur in old plants with ripe seeds, it is present only in the younger stages of development.

It is hardly necessary to point out the economic importance of this occurrence of cyanogenetic glucosides in the younger stages of plants like *Lotus arabicus* and *Sorghum;* much loss of stock has been sustained by their consumption by cattle.

### SAPONINS.

According to the researches of Greshoff,† the saponins are very widely distributed in the higher plants; he has identified them in various plants belonging to the natural orders: Piperaceæ, Saururaceæ, Primulaceæ, Loganiaceæ, Oleaceæ, Polemoniaceæ, Proteaceæ, Caprifoliaceæ, Compositæ, Cucurbitaceæ, the majority of the natural orders of the cohort Centrospermæ, Ranunculaceæ, Magnoliaceæ, Leguminosæ, Rosaceæ, Saxifragaceæ, Pittosporaceæ, Polygalaceæ, Rutaceæ, Rhamnaceæ, Guttiferæ, Thymelæaceæ, Combretaceæ, Myrtaceæ, Lecythidaceæ, Araliaceæ, Gramineæ, Liliaceæ, and Gleicheniaceæ.

The term saponin, though originally restricted to a specific

* Dunstan, Henry and Auld: "Proc. Roy. Soc., Lond.," B., 1906, **78**, 145, 152.

† Greshoff: "Kew Bulletin," 1909, 397; for summary of work on saponins see Winterstein and Maxim: "Helv. chim. Acta," 1919, **2**, 195.

substance obtained from the root of *Saponaria rubra* and *S. alba* is now applied to a large group of compounds, all of which have properties similar to those possessed by the original saponin.

### *General Properties and Uses of Saponins.*

The saponins are mostly amorphous colloidal substances which dissolve readily in water; their aqueous solutions, if shaken up alone, produce a froth, but if shaken in the presence of oils, fats or resins, they produce emulsions which are characterized by their great stability.

Connected with their emulsifying property is the employment of saponins as substitutes for soaps, a fact which is indicated in the name Saponin itself and also by the names *Saponaria*, soap wort and Quillaia (meaning wash wood), etc.

The so-called soap nuts are the fruits of *Sapindus* (fructus saponis indici) and these, as well as the beans of *Entada scandens* and *Lychnis chalcedonica* or Tartary soap, are largely used in the East for washing clothes, since they have no deleterious effect on the colour or the fibre of the most delicate fabrics.

Aqueous solutions of saponins have a marked power of retaining dissolved gases, as, for example, carbon dioxide; for this reason saponins are occasionally added to effervescent drinks, such as ginger-beer or lemonade, a use which is to be deprecated owing to their toxic properties.*

Occasionally saponins are employed for making suspensions of solids in water since they exert an inhibiting effect on the precipitation or deposition of suspended solids. Concentrated aqueous solutions of the saponins have adhesive properties.

### *Solubility.*

The saponins are, as a rule, neutral substances which dissolve readily in water, but a few are acid in character and require a small quantity of alkali to enable them to dissolve completely.

* The saponin obtained from the bark and wood of *Guajacum officinale* is occasionally used for this purpose since it is practically non-poisonous, its hæmolytic action (see p. 183) being only very slight.

From their aqueous solutions saponins may be precipitated unchanged by the addition of ammonium sulphate.

In the form of lead or barium compounds they may be precipitated from aqueous solutions by the addition of either lead acetate or basic acetate of lead or by means of a solution of barium hydrate.

The saponins are almost all insoluble in absolute alcohol, ether, chloroform and benzene.

### *Physiological Action.*

The saponins are characterized by their strongly marked toxic properties. Fishes, particularly, are very sensitive to saponins, being killed by a solution of one part in 100,000 parts of water, a fact which is made use of by fishermen in the East, as the fish killed by these means are not unfit for human consumption.

Saponins have a powerful solvent action on blood corpuscles, a property which is known as hæmolysis. This property may be connected with their tendency to combine with cholesterol,* which substance they abstract from the blood corpuscles thereby rendering them soluble.

The action may be illustrated by adding a small quantity of a solution of a saponin † in 0·9 per cent sodium chloride to 1 c.c. of a solution made by dissolving 1 c.c. of defibrinated blood in 100 c.c. of 0·9 per cent sodium chloride; after a short time the blood corpuscles will have dissolved leaving a clear solution.

The hæmolytic action may be destroyed by shaking up some of the saponin solution with an ethereal solution of cholesterol and then warming for some hours at 36° C.; this treatment causes the saponin to combine with cholesterol to produce an inactive compound which has no solvent action on blood corpuscles.

### *Chemistry of the Saponins.*

As already stated, the majority of saponins are neutral substances, while a few have feebly acid properties. Only a

* They also combine with phytosterol.

† The saponins of *Guajacum officinale* and *Bulnesia Sarmienti* have hardly any hæmolytic action, and hence are only slightly toxic.

single saponin, namely, Solanin, has basic properties; this substance, which occurs in *Solanum nigrum*, *S. dulcamara* and in the fruit of potatoes, owes its basic property to the presence of a nitrogen atom (see Nitrogen Bases, p. 261), and appears to form a connecting link between the saponins and the alkaloids.

The neutral saponins are precipitated from solution by basic lead acetate, while acid saponins are precipitated by lead acetate. Similarly, barium hydroxide precipitates neutral saponins in the form of their barium compounds (see below).

On hydrolysis with dilute mineral acids* the saponins yield sugars such as glucose, galactose, arabinose, and rhamnose, together with other substances termed *sapogenins*, the constitution of which is unknown.

The nature of the sapogenin obtained from any particular saponin varies with the conditions of the hydrolysis; in some cases careful hydrolysis may yield a primary sapogenin and a sugar, while more complete hydrolysis gives rise to an end sapogenin together with more sugar.

The hydrolysis of Digitonin, the saponin contained in *Digitalis purpurea*, may, according to Kiliani, be represented by the equation :—

$$\underset{\text{Digitonin}}{C_{54}H_{92}O_{28}} + 2H_2O = \underset{\text{Digitogenin}}{C_{30}H_{48}O_6} + 2\underset{\text{Glucose}}{C_6H_{12}O_6} + 2\underset{\text{Galactose}}{C_6H_{12}O_6}$$

On mixing together alcoholic solutions of a saponin and of cholesterol a precipitate of the cholesterol compound is at once formed (p. 17). These cholesterol compounds are, as a rule, easily decomposed; in most cases, prolonged extraction with ether will remove the cholesterol, and the saponin is recovered unchanged and possesses its original physiological action.

The saponins are reducing agents, and will reduce ammoniacal silver nitrate to metallic silver; similarly, prolonged boiling with mercuric chloride reduces this substance to calomel; saponins also blue a solution of potassium ferricyanide containing ferric chloride, by reducing the ferric salt

* Hydrolysis can, in some cases, be effected by bacteria, and Quillaia saponin is even said to be hydrolysed by emulsin (see Gonnermann: "Pflüger's Archiv," 1906, 113, 185).

to the ferrous condition, and so giving rise to the formation of Turnbull's blue.

If boiled with acetic anhydride, alone or in presence of sodium acetate or zinc chloride, the saponins are converted into acetyl derivatives which are no longer toxic. On boiling the acetyl derivatives with alcoholic potash the acetyl groups are removed, but the resulting compound is not identical with the original saponin.

When treated with a hot saturated solution of baryta a saponin is precipitated in the form of a barium compound. If this latter is treated with the requisite amount of sulphuric acid the barium may be completely removed, but the resulting substance, unlike the original saponin, is physiologically inactive.

*Reactions.*

The following reactions are made use of in demonstrating the presence of a saponin :—

1. Aqueous extracts readily form a froth when shaken up.
2. Concentrated sulphuric acid gives with all saponins, either in the cold or on warming, a violet or red colour.
3. Concentrated sulphuric acid containing a little ferric chloride gives with many saponins a blue or bluish-green colour or fluorescence.
4. The hæmolytic action described on page 183 may be tried.

Although the above reactions are best carried out in the test tube, numbers 2 and 3 may be made use of in microchemical work.

## OTHER GLUCOSIDES.

In addition to the above, there occur in plants a large number of other glucosides which do not readily lend themselves to reasonable classifications. The exigencies of space will permit of reference only to the following, which are among the more important and more interesting of them.

### SINIGRIN.

Sinigrin, or myronate of potash, occurs in the seeds of certain Cruciferæ, notably *Sinapis nigra.*

*Preparation.*

Green gives the following method for its extraction: One kilogram of the seeds of the black mustard is ground to a fine powder, and then extracted with one and a half litres of 82 per cent alcohol. The mixture is heated on a water bath until the volume of the alcohol is reduced by about one-sixth, the alcoholic extract is then filtered off, and the residue pressed while it is still hot. The operation is then repeated; the residue is dried at 100° C., and digested for twelve hours with eight times its volume of cold water. A small quantity of barium carbonate is added to this aqueous extract, which is then evaporated to a syrup. The sinigrin is contained in this syrup, and is extracted by boiling with 82 per cent alcohol. Finally, the alcoholic extract is evaporated down, when the glucoside crystallizes out in rhombic prisms, which are freely soluble in water and warm alcohol, but much less soluble in cold.

Sinigrin is split by the enzyme myrosin into glucose, potassium hydrogen sulphate and allyl isothiocyanate, or mustard oil, which may be recognized by its distinctive smell.

$$C_{10}H_{18}O_{10}NKS_2 = C_6H_{12}O_6 + KHSO_4 + CH_2:CHCH_2NCS$$

CONIFERIN.

This glucoside occurs in various coniferous trees, especially in young parenchyma, and also in asparagus. With concentrated sulphuric acid coniferin gives a violet coloration, while hydrochloric acid and phenol give a blue coloration. Most reagents used in the demonstration of the lignification of cell walls (p. 164) give similar reactions both with coniferin and vanillin, and for this reason it is supposed that both these substances occur in such thickened walls. By using a mixture of thallin sulphate (which stains vanillin yellow) with thymol (which with coniferin and concentrated hydrochloric acid gives a blue colour), it has been shown that coniferin is more abundant in young wood cells, whilst vanillin occurs more extensively in the older elements.

Such colour reactions must, however, be used with caution since they mostly depend on the presence of some complex

## ERRATUM.

Page 186. *The equation should read :—*

$$C_{10}H_{16}O_9NKS_2 + H_2O = C_6H_{12}O_6 + KHSO_4 + CH_2:CHCH_2NCS$$

*And the footnote on page* 167 *should be deleted.*

capable of furnishing an aldehydic group and the same reactions are likewise given by salicylic and cinnamic aldehydes, while coniferin contains no aldehyde group but yields vanillin on oxidation.

Coniferin crystallizes in needle-shaped crystals, m.p. 185°, and is soluble in warm water and warm alcohol. On hydrolysis by mineral acids or by emulsin it gives glucose and coniferyl alcohol:—

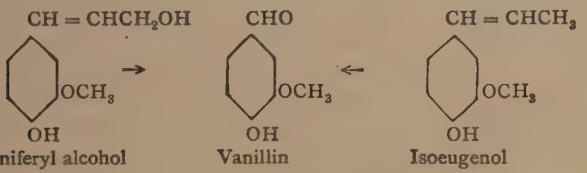
$$\underset{\text{Coniferin}}{C_{16}H_{22}O_8} + H_2O = C_6H_{12}O_6 + \underset{\text{Coniferyl alcohol}}{C_{10}H_{12}O_3}$$

The latter is a crystalline substance melting at 73°.

Both coniferin and coniferyl alcohol when oxidized with potassium bichromate and sulphuric acid yield vanillin, the aromatic constituent of the fruits of *Vanilla planifolia*.

The reaction was formerly employed for the preparation of artificial vanillin, but has now been replaced by the oxidation of isoeugenol, which is obtained by the action of dilute alkalis upon eugenol, a substance contained in oil of cloves.

The relationship between these three substances is as follows:—




Coniferyl alcohol — Vanillin — Isoeugenol

## SALICIN.

Salicin, $C_{13}H_{18}O_7$, occurs in the bark of *Salix viminalis*. It has a bitter taste and crystallizes in colourless prisms and scales. It is sparingly soluble in cold water but is more soluble in hot alcohol, especially amyl alcohol, and may be extracted from aqueous solutions by means of this solvent. Microscopically, salicin is indicated by the fact that it gives a bright red colour with strong sulphuric acid, also with Fröhde's reagent * it yields a violet coloration. On steeping the section in a solution of emulsin, saligenin is produced which gives a blue colour with ferric chloride.

* Sodium molybdate dissolved in concentrated sulphuric acid.

*Preparation.*

Salicin may be prepared by boiling the willow bark with water which will extract a certain amount of tannin, colouring and other matters together with the salicin. The greater part of impurities may be precipitated by the addition of lead acetate. The precipitate is then filtered and a stream of sulphuretted hydrogen is passed through the filtrate in order to remove the lead. The filtrate on evaporation yields crystals of salicin which may be further purified by recrystallization from alcohol.

Another method is to treat the bark with benzene in order to remove resinous substances, colouring matters, etc., and then digest with alcohol (sp. gr. ·85). The solution thus obtained will contain the glucoside and tannin; the latter substance may be removed by precipitation with hide powder or with gelatine. The filtrate will contain the salicin, which on evaporation and cooling will be deposited in the form of crystals.

Salicin is hydrolysed by emulsin to glucose and the alcohol saligenin according to the following equation :—

$$\underset{\text{Salicin}}{C_{13}H_{18}O_7} + H_2O = C_6H_{12}O_6 + \underset{\text{Saligenin}}{C_6H_4OH\,CH_2OH}$$

By the action of sulphuric acid and potassium bichromate salicin is oxidized to salicylic aldehyde $C_6H_4OHCHO$; this substance is a fragrant colourless liquid, b.p. 196°, which occurs in the essential oil of *Spiræa Ulmaria;* it is soluble in water, the solution giving an intense violet coloration with ferric chloride; salicylic aldehyde stains the skin yellow.

By employing dilute nitric acid as the oxidizing agent, salicin is converted into helicin, a glucoside which on hydrolysis yields glucose and salicylic aldehyde :—

$$\underset{\text{Salicin}}{C_{13}H_{18}O_7} + O = \underset{\text{Helicin}}{C_{12}H_{16}O_7} + H_2O$$

$$\underset{\text{Helicin}}{C_{12}H_{16}O_7} + H_2O = C_6H_{12}O_6 + \underset{\text{Salicylic aldehyde}}{C_6H_4OHCHO}$$

The investigations of Weevers tend to show that ordinarily in the decomposition of salicin, saligenin is really an intermediate substance, the ultimate products being glucose and catechol. Thus salicase splits salicin into glucose and sali-

genin, saligenase produces catechol from the saligenin, and when the leaves decay a third enzyme, catecholase, produces from the catechol an amorphous black pigment. He found that in places where depletion of salicin was taking place the saligenin appeared in quantities insufficient in amount to account for the whole of the salicin; also that catechol occurred in such places, after the glucoside had disappeared, in a sufficiently large quantity to warrant the above conclusion. Weevers considers that when glucose and catechol are produced the sugar is translocated, whilst the catechol remains *in situ*, and combines with fresh glucose, and so reconstructs salicin.

## INDICAN.

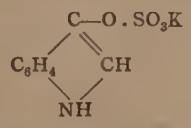
Indican,* $C_7H_6NC.O.C_6H_{11}O_5$, is the name given to a glucoside which occurs not only in *Indigofera anil*, *I. arrecta*, *I. tinctoria*, and *I. sumatrana*, but also in other plants, such as *Isatis tinctoria*, *Polygonum tinctorium*, species of *Phajus* and other orchids, e.g. *Calanthe* and *Strobilanthes*. In the plant, indicane is well distributed in the aerial organs. Thus in *Indigofera*, it is found in all the tissues of the leaf except the tracheæ of the xylem, it is also abundant in the apex of the stem in all tissues except the wood vessels and the laticiferous system. The flowers also have a small quantity, but the root is characterized by its absence.†

At one time it was considered that the chloroplasts played an important direct part in the formation of indicane, but Leake can find no evidence of this.

### *Identification.*

1. The tissue may be boiled in a 2 per cent solution of

* The name indican is also applied to a compound of the formula




This substance, which is more correctly described as indoxyl potassium sulphate, occurs in small quantities in human urine and also in the urine of herbivora.

† Leake: "Ann. Bot.," 1905, 19, 297.

ammonia. The addition of chloroform to the filtered extract may be made to separate the indigo; the chloroform will sink to the bottom of the solution, carrying with it the indigo.

2. Tissues containing indican on exposure to the vapour of alcohol for twenty-four hours will turn blue; the reaction will be better marked if the chlorophyll be subsequently dissolved out with absolute alcohol.

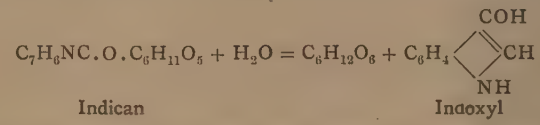
3. The tissue, in bulk or in section, may be boiled in strong hydrochloric acid and ferric chloride added. The indigo will separate out.

4. The tissue is cut up into pieces and quickly immersed in the following mixture:—

| | |
|---|---|
| Glacial acetic acid | 2 c.c. |
| Strong sulphuric acid | 1 c.c. |
| Ammonium persulphate | ·5 gram. |
| Water to | 100 c.c. |

As this fluid penetrates the cells, the indigo is precipitated in blue granules. When penetration is effected fully, the material is washed for twenty-four hours in water, after which sections may be cut and stained in the usual way.

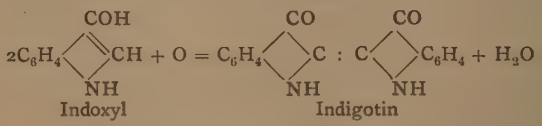
Indican is hydrolysed by indimulsin, with which it is associated in the plant, into glucose and indoxyl according to the equation:—




Indican Indoxyl

The same reaction can also be effected, though more slowly, by emulsin.

The resulting indoxyl, by exposure to air, is oxidized to the deep blue colouring matter indigotin.




Indoxyl Indigotin

The production of indigotin from the indigo plant is based on these two reactions and consists in fermenting the plant material by steeping it in slightly acidified water for a few hours, and then exposing to the air the fermented extract to which a little ammonia has been added to facilitate oxidation.

Prepared in this way the natural indigo contains, in addition, to indigotin, varying proportions of indirubin (a red colouring matter), indigo brown, etc., produced as by-products in the oxidation of the indoxyl.

Until a few years ago, *Indigofera* was the only source of the blue colouring matter indigo, for the obtaining of which large tracts of country were under cultivation in India. Within recent years, however, the natural production of indigo has suffered from very severe competition with the synthetic product and the planters have been compelled to improve their output. The importance of attention to fertilizing the soil has been shown by the fact that superphosphate manuring has considerably increased the yield and improved the quality of the resulting indigo.*

* Davis: "Agric. Res. Inst. Pusa.," Indigo Publ., No. 4, 1918.

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Armstrong: "The Simple Carbohydrates and Glucosides," London, 1919.
Henry: "Science Progress," 1906, 1, 39.
Kobert: "Beiträge zur Kenntniss der Saponinsubstanzen," Stuttgart, 1904.
Robinson: "Science Progress," 1909, 3, 575.
Van Rijn: "Die Glykoside," Berlin, 1900.

# SECTION V.

## TANNINS.

THE term Tannin is variously employed by different writers, sometimes to denote a particular substance better described as gallotannic or digallic acid, and sometimes as a collective term for a whole group of substances having certain characteristics in common. In order to prevent confusion it is proposed here to use the word tannin only in the latter sense.

The properties of the tannins may be summarized as follows:—

1. They are mostly uncrystallizable colloidal substances with astringent properties.

2. They precipitate gelatine from solution and form insoluble compounds with gelatine yielding tissues, a property which enables them to convert hide into leather.*

3. They all give blackish-blue or blackish-green colours with ferric salts, a fact which is made use of in the manufacture of ink.

4. They are precipitated from solution by many metallic salts such as copper or lead acetates or stannous chloride, etc.

5. They are precipitated from solution by a strong aqueous solution of potassium bichromate or by a 1 per cent solution of chromic acid.

* According to some authors this property is not an essential characteristic of tannins; on the other hand Dekker prefers to regard those substances which do not give this reaction as pseudo-tannins and includes under this heading caffetannic acid and the tannins of *Portlandia grandiflora*, *Asperula odorata*, *Rubia tinctorum*, *Scrophularia nodosa*, *Humulus Lupulus*, etc. Similarly Procter points out that such substances as moringatannic acid, or maclurin, and caffetannic and lupulotannic acids, are more closely related to the colouring matters than to the tannins; maclurin which is a pentahydroxybenzophenone is the yellow colouring matter occurring in the substance known as fustic, obtained from the wood of *Morus tinctoria* (see formula on p. 202).

6. They precipitate from solution both alkaloids and substances of a basic nature, such as basic organic colouring matters.

7. In alkaline solution the tannins, and many of their derivatives, readily absorb oxygen, becoming dark in colour.

8 With a solution of potassium ferricyanide and ammonia they give a deep red colour.

It must be borne in mind, however, that none of these reactions, taken separately, are specific for tannins; they may be given by many other substances as well, but all true tannins answer them as a whole.

## OCCURRENCE.

Tannin, using the word as a generic term, is generally looked upon as an aplastic substance, and is very widely distributed in the vegetable kingdom.

In certain Algæ, e.g. *Spirogyra*, *Mesocarpus* and *Zygnema*, it occurs in the cells in the form of numerous small vesicles; in the Fungi, tannin is stated to be more abundant in parasites than in saprophytes, thus hardly any occurs in the Agaricineæ whilst in the Polyporeæ it is present in much larger amounts.

In the higher plants it occurs more or less generally throughout a tissue, for example in bark, or it may be restricted, in the more mature parts, to special cells which may be isolated or superposed one above the other in the form of chains.

Amongst the higher plants there is no great phylum in which tannin is not found; it occurs in the ferns, e.g. *Angiopteris* and *Aspidium;* in Gymnosperms, e.g. *Pinus;* and also in innumerable Angiosperms, in all parts.

Thus it obtains in the roots of *Trianea*, *Desmanthus* and *Pistia;* in the stems, where it may be accumulated, especially in the bark, of *Quercus*, many species of *Cæsalpinia*, *Eucalyptus occidentalis*, *Castanea* and *Humulus;* in the leaves of *Cerasus*, *Rhus*, *Ficus*, and *Rhododendron;* in the fruit, especially if unripe, of *Terminalia Chebula*, *Cæsalpinia coriaria*, *Pyrus*, and *Phaseolus;* and more rarely in the seeds, either before or after germination, of *Areca Catechu*, *Echium vulgare* and other Boraginaceæ.

Further, tannin is often found in more or less special structures, e.g. the cells of the pulvini of *Mimosa pudica* and *Robinia pseudacacia;* in the gland cells of *Sarracenia* and *Utricularia;* in the hairs of *Primula* and *Hedera;* and also in laticiferous tissue.*

Finally, it may be remarked that it is especially abundant in pathological growths such as galls, which may contain from 25 to 75 per cent of tannin.

Kraemer† has investigated the galls formed by the agency of *Cynips aciculata*, a gall fly, upon *Quercus coccinea.* He found that during the chrysalis stage gallic acid was produced, probably at the expense of the starch, and as the imago developed the gallic acid gave place to tannic acid.

In the cell, the tannin occurs in solution in the cell sap, and since tannin forms a precipitate with albuminous matter it follows that the layer of protoplasm around the tannin vesicles must be impermeable to it; if this were not so the protoplasm would be tanned on the production of tannin.

Economically tannin is of great value although it is perhaps not so extensively used at the present day as in the past. As is well known its principal use is in the making of leather‡; in this connexion salts of chromic acid have come into use as a substitute, especially in the manufacture of the cheaper grades of leather. Formerly tannins were almost exclusively used in the manufacture of black ink, whereas at the present time various preparations of aniline dyes are in vogue. Among other uses for tannins may be mentioned their value for medicinal purposes and their use as mordants in certain dyeing operations.

The chief sources of tannins are the bark or the wood of various species of *Acacia*, *Castanea*, *Eucalyptus*, and *Quercus;* the bark of the mangrove *Rhizophora Mangle*, the roots of *Rumex hymenosepalus* (Canaigre) and the leaves of *Rhus Coriaria* (Sumach).

Gallotannic acid is obtained from galls, especially the galls which occur on *Quercus lusitanica;* but the galls on other species of oak, e.g. *Q. sessiliflora*, *Q. pedunculata*, *Q. Ilex*, *Q.*

* For details of the distribution of tannin in *Ribes*, etc., see Dekker: "Rec. trav. bot. néerlandais," 1917, 14, 1.

† Kraemer: "Bot. Gaz.," 1900, 30, 274.

‡ See Procter: "The Making of Leather," Cambridge, 1914.

*Cerris*, *Q. Coccinea*, etc., and other plants, e.g. species of *Tamarix*, are used to a greater or lesser extent.

The amount of tannin present in certain plants varies according to the physiological state, the season of the year, and the conditions of growth.

In *Pinus* it is stated that the amount of tannin varies with that of the resin; thus in the spring it was found that as the tannin decreased in amount so the resin increased. Peacock * found that in *Heuchera americana* the tannin was most abundant in October and least in May, whilst the amount of starch present was greatest in March. Trimble and Peacock found that in *Geranium maculatum* the maximum amount of tannin obtained in April, i.e. just before the period of flowering. From this phase onwards there was a gradual decrease until the minimum was reached in October.

It is found that the more vigorous trees yield the most tannin, and that the character of the soil appears to be of importance. It has been found that oak trees grown in a poor dry soil yield a bark richer in tannin than those grown on the soil of damp lowlands.

According to the observations of Henri, a calcareous soil is more beneficial with regard to tannin formation than is a siliceous soil.

It is not impossible that the different yields of tannin given by the same plant grown in different situations may be due to the relative abundance of the mineral food-materials; thus it has been found that in some instances, e.g. in *Spirogyra* and *Phaseolus multiflorus*, the formation of tannin is inhibited by the absence of chlorine.

With regard to seasonal variation in the amount of tannin in the bark of the oak, the following estimations are given by Eitner:—†

| | *Q. pedunculata.* | *Q. sessiliflora.* |
|---|---|---|
| April | 14·8 per cent | 12·86 per cent |
| May | 10·71 „ | 10·46 „ |
| June | 12·33 „ | 10·58 „ |
| July | 9·8 „ | 8·11 „ |
| August | 11·23 „ | 10·74 „ |

* Peacock: "Amer. Journ. Pharm.," 1891, 172.
† Eitner: "Der Gerber, Vienna," 1878, 4.

For the inner bark of the American oak, *Quercus Prinus*, Trimble * found the following seasonal variation :—

| | |
|---|---|
| December . . . . . | 9·33 per cent |
| March . . . . . | 10·63 „ „ |
| June . . . . . | 11·22 „ „ |
| July . . . . . | 11·70 „ „ |
| September . . . . . | 6·66 „ „ |

As a general rule the barks collected in May and June are the richest in tannin, but this does not hold for all parts of plants. Thus, Levi and Wilmer † found that in the case of the horse-chestnut, *Aesculus Hippocastanum*, the youngest leaves were richest in tannin, the minimum amount obtained in June, whilst in August the quantity rapidly rose until the original value was reached; finally a diminution of tannin occurred just before leaf-fall. Weekly analyses of leaves were made from the opening of the buds to the fall of the leaves in September. The obtained percentages of tannin were: 6·5, 3·3, 3·5, 2·8, 3·7, 3·2, 1·9, 2·8, 3·5, 3·6, 3·4, 5·1, 3·1, 5·3, 4·4, 4·3, 3·4, 6·2, 6·6, 5·2, 6·1, 6·5, 4·5 per cent.

These variations in the tannin-content of parts of plants are of great interest; the value, however, of such estimations would be greatly enhanced if estimations were carried out at the same time to see whether, for instance, there is any obvious relationship between the tannin-content of leaves and of other parts of the plants such as the periderm.

## MICROCHEMICAL REACTIONS OF TANNINS.

Before passing on to the detailed examination of the various tannins, the following microchemical tests may be mentioned, but it must be borne in mind that these reactions do not enable one to distinguish between the various tannins.

1. Tannins reduce Fehling's solution.

2. They are precipitated by basic lead acetate and the salts of many other metals; thus uranium acetate gives a brown precipitate or a brown or brown-red coloration, and an aqueous solution of copper acetate gives a brown precipitate.

3. Potassium bichromate in a strong aqueous solution or

* Trimble: "The Tannins," Philadelphia, 1892, 1894.
† Levi and Wilmer: "Hide and Leather," 1905.

a 1 per cent solution of chromic acid gives brownish-coloured precipitates.

4. A red-brown to brown coloration is obtained by the use of a dilute ammoniacal solution of potassium ferricyanide. This test is very delicate, and the reagent must be used sparingly since the coloration is destroyed by an excess.

5. The addition of a neutral solution of ferric chloride gives a blue black or greenish coloration or precipitate. Moeller recommends the use of a solution of anhydrous ferric chloride in anhydrous ether.

6. A solution of ammonium molybdate in a strong solution of ammonium chloride gives a copious yellow precipitate with many tannins; when added to digallic acid a red coloration results. According to Gardiner* this reagent affords a means of distinguishing glucoside tannin from tannic acid.

The red yellow colour obtained by adding ammonium molybdate to tannic acid is destroyed by oxalic acid.

7. Lime water gives a white precipitate which turns red, brown or blue.

8. Aqueous solutions of various organic bases such as caffeine and antipyrin precipitate the tannins.

van Wisselingh† recommends 1 per cent aqueous solutions of antipyrine and of caffeine.

It must be remembered that several other substances besides tannins are precipitated by these reagents.

9. Pfeffer has drawn attention to the fact that tannins are precipitated by methylene blue without prejudice to the vitality of the cells. The stain must be used in very dilute solutions (1 pt. in 500,000 of water), and the tissue under investigation must remain in a large quantity of the solution for several hours. Van Wisselingh's experience is contrary to Pfeffer's, for he finds that even very dilute solutions of methylene blue are harmful to *Spirogyra*, the plant used by Pfeffer, and after treatment for several days only a little of the tannin was precipitated.

10. On the addition of a solution of gelatine a dirty white precipitate is formed.

*Gardiner: "Proc. Camb. Phil. Soc.," 1883, 4, 387.
†van Wisselingh: "Konin. Akad. v. Wetensch., Amsterdam," 1910, 685.

11. A brilliant red colour, even when the tannins are in a very dilute solution, results from the addition of an aqueous solution of iodine in potassium iodide mixed with a little 10 per cent ammonia.

12. According to Moore,* the action of Nessler's solution (a saturated solution of mercuric iodide in a solution of potassium iodide and potash) varies.

(*a*) A brown precipitate is formed immediately, e.g. epidermis of primrose leaf.

(*b*) A yellow colour is produced which turns reddish brown; finally a brown precipitate comes down, e.g. stem of Yew and *Aucuba japonica.*

(*c*) A yellow coloration. The compound produced readily diffuses through the cell wall, e.g. young stem and hairs of the ivy.

The following are microchemical tests for gallic acid :—

1. The rapidity of the reaction with potassium chromate may provide a means of distinguishing gallic acid from tannic acid, for in the case of the former a precipitate immediately comes down, whilst in the case of tannic acid, according to Drabble and Nierenstein, the reaction is either very slow or entirely negative.

2. Potassium cyanide in aqueous solution gives a pink coloration with gallic acid.

3. With Nessler's solution gallic acid gives a grey-green precipitate.

With this same reagent pyrogallol immediately yields a brown precipitate; pyrocatechol forms a deep green precipitate which changes to greenish brown; and a dirty green precipitate is given by protocatechuic acid.

Vinson† recommends exposing the material to be examined to the vapour of nitrous ethers in order to fix and stain the tannin in vegetable tissues. There is, ordinarily, no necessity to cut up the tissue, and as the tannin is deposited in the cells in which it occurs, the method is very convenient for tracing the distribution of the substances in question. The resulting colour varies; thus, the juice of unripe grapes gives a dense brown precipitate; the juice of the persimmon a wine-

* Moore: "Journ. Linn. Soc., Lond., Bot.," 1891, **27**, 527.
† Vinson: "Bot. Gaz.," 1910, **49**, 222.

red coloration; tannic and gallic acids yield a yellow tint, phloroglucin red, and so on.

The material to be examined is merely exposed to the vapour of ordinary sweet spirits of nitre which contain 4 per cent of the ethyl nitrate, or a 20 per cent alcoholic solution of the commercial nitrous ether may be employed. If the latter method be used the time required for full precipitation is considerably less.

### CHEMISTRY.

We have as yet comparatively little certain knowledge concerning the chemical constitution of even the simplest tannins. Thus, for example, although ordinary tannic or gallotannic acid is generally regarded as an anhydride formed by the removal of one molecule of water between two molecules of gallic acid, according to the equation

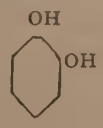
$$\underset{\text{Gallic acid}}{2C_7H_6O_5} - H_2O = \underset{\text{Gallotannic acid}}{C_{14}H_{10}O_9}$$

there are even now, as will be seen below (p. 213), differences of opinion with regard to the exact position from which the two atoms of hydrogen and one of oxygen have been removed.

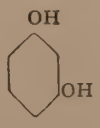
While the composition of the various classes of tannins of course varies considerably, they are probably all more or less complex derivatives of gallic or ellagic acids, or their methylated derivatives, or are condensation products of these or similar acids, with various phenolic substances.

In view of these facts the classification and properties of the tannins will be more easily understood if preceded by a brief description of certain relatively simple phenolic substances from which the complex tannins are built up (p. 219). The substances include the following:—

1. The Dihydric phenols—pyrocatechol, resorcinol and hydroquinone.



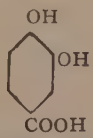
Pyrocatechol



Resorcinol

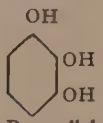
Hydroquinone

2. The dihydroxy acid—Protocatechuic acid.

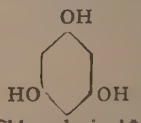



Protocatechuic acid

3. The Trihydric phenols—Pyrogallol and phloroglucinol.

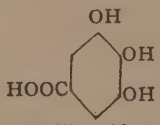



Pyrogallol




Phloroglucinol *

4. The trihydroxy acid—Gallic acid.



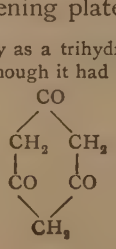

Gallic acid

The above substances occur in varying proportions among the products obtained by subjecting different tannins to fusion with caustic potash or other chemical treatment; and upon their occurrence is based the chemical classification of the tannins.

PYROCATECHOL, CATECHOL OR PYROCATECHIN. $C_6H_4(OH)_2$.

This substance is so called from the fact that it is obtained by the destructive distillation of catechu, an extract of the bark of *Mimosa Catechu;* it is also obtained by the fusion with potash of other tanno-resins such as kino, the sap of various species of *Pterocarpus*, *Butea* or *Eucalyptus;* also it occurs in small quantities combined with sulphuric acid in the urine of horses and of human beings. It crystallizes from benzene in colourless glistening plates and melts at 140°.

* Although behaving normally as a trihydric phenol, phloroglucinol reacts with certain ketonic reagents as though it had the following constitution:—



*Reactions.*

1. Pyrocatechol is precipitated from aqueous solution by lead acetate. (Distinction from resorcin and hydroquinone.)

2. With ferric chloride it gives a green colour which is changed to violet on the addition of sodium acetate.

3. Like pyrogallol it reduces silver nitrate in the cold and has therefore been used as a photographic developer.

4. It reduces Fehling's solution on warming.

RESORCINOL. $C_6H_4(OH)_2$.

This is isomeric with pyrocatechol (for constitutional formula see page 199); it does not generally occur in tannins* but in certain resins, notably galbanum resin and asafœtida.

It is used commercially in the manufacture of dye-stuffs, and when heated with sodium nitrite gives the indicator known as Lacmoid.

Resorcinol crystallizes from benzene in colourless needles and melts at 119°; it is somewhat soluble in water, the solution having a sweetish taste.

*Reactions.*

1. It is not precipitated from solution by lead acetate.

2. With ferric chloride it gives a dark violet colour which is destroyed by the addition of sodium acetate.

3. It reduces ammoniacal silver nitrate or Fehling's solution on warming.

HYDROQUINONE.

This third isomer of the formula $C_6H_4(OH)_2$ likewise is not found in tannins, but occurs combined with glucose in the glucoside arbutin and uncombined in the leaves and flowers of *Vaccinium Vitis Idæa*. Hydroquinone crystallizes from water in colourless prisms and melts at 169-170°.

*Reactions.*

1. It gives no precipitate with lead acetate.

2. Ferric chloride gives no colour but oxidizes it to quinone.

* According to Nierenstein, it is produced together with protocatechuic acid and phloroglucinol from quebracho tannin by potash fusion.

3. It reduces ammoniacal silver nitrate and Fehling's solution.

4. It turns brown in alkaline solution when exposed to the air; its powerful reducing properties enable it to be used in photography as a developer.

### PROTOCATECHUIC ACID.

Protocatechuic acid is closely related to pyrocatechol, differing from this substance only by one atom of carbon and two of oxygen which it loses when heated above its melting point (199°), thus:—

$$\underset{\text{Protocatechuic acid}}{C_6H_3(OH)_2COOH} = \underset{\text{Pyrocatechol}}{C_6H_4(OH)_2} + CO_2$$

It rarely occurs uncombined except, for example, in the fruits of *Illicium religiosum;* in combination, it is found in such substances as Catechin and Maclurin,* both of which give protocatechuic acid on potash fusion; it may further be obtained by a similar process from many resins such as gum benzoin, asafœtida, myrrh and also from kino.

Finally its dimethyl ether, known as veratric acid, $C_6H_3(OCH_3)_2COOH$, occurs together with the alkaloid veratrine in the seeds of *Veratrum sabadilla.*

Protocatechuic acid is soluble in water and melts at 199°.

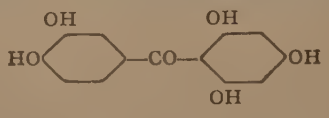
#### *Reactions.*

1. Aqueous solutions of protocatechuic acid are precipitated by lead acetate.

2. It gives a green colour with ferric chloride; on addition of very dilute sodium carbonate the green colour changes first to blue and then to red.

3. Ferrous salts produce with protocatechuic acid a violet colour.

* Maclurin, sometimes also called moringatannic acid, is a colouring matter of fustic; its constitution is represented by the formula



### PYROGALLOL OR PYROGALLIC ACID. $C_6H_3(OH)_3$.

This substance is so called because it is formed by heating gallic acid according to the reaction—

$$\underset{\text{Gallic acid}}{C_6H_2(OH)_3COOH} = \underset{\text{Pyrogallol}}{C_6H_3(OH)_3} + CO_2$$

It is also formed by fusing hæmatoxylin with caustic potash.

Pyrogallol crystallizes in colourless needles or plates melting at 132° and is soluble in water; its solution, in excess of caustic alkali, absorbs oxygen with great avidity, turning brown and forming carbon dioxide, acetic acid and other substances.

Pyrogallol reduces salts of silver, mercury, or gold to their respective metals.

*Reactions.*

1. Pyrogallol is precipitated from solution by lead acetate but not by lead nitrate.

2. It gives a blue colour with ferrous sulphate and a red colour with ferric chloride.

3. Aqueous or alcoholic solutions of pyrogallol, in common with those of gallic acid or tannic acid, are coloured purple by iodine.

4. Lime water added to an aqueous solution of pyrogallol produces a purple colour which rapidly becomes brown.

5. Solutions of pyrogallol give no precipitate with gelatine.

6. Potassium cyanide gives a reddish-brown coloration, which turns brown, but the red tint becomes apparent again on shaking.

### PHLOROGLUCINOL. $C_6H_3(OH)_3$.

Phloroglucinol, which is isomeric with pyrogallol, is produced by fusing a number of resins, such as catechin, kino, dragon's blood, etc., with potash, and it occurs naturally, in a number of glucosides, such as phloretin, quercetin, hesperidin, etc. It crystallizes with two molecules of water, but loses them if heated to 100°, and melts at 218°; it dissolves readily in water, forming a sweet solution, and is readily soluble in alcohol or ether.

*Reactions.*

1. Phloroglucinol is precipitated from solution by lead acetate.

2. It gives with ferric chloride a bluish-violet colour.

3. A solution of phloroglucinol in hydrochloric acid produces a red colour on a pine wood shaving; this reaction can also be made use of for detecting lignified cell walls (p. 164).

4. It is a reducing agent, and reduces Fehling's solution.

In addition to the above-mentioned phenols, which are products of the decomposition of tannins by heat or by fusion with alkalis, there are other important substances produced by acid hydrolysis, namely, gallic and ellagic acids and the phlobaphenes.

### GALLIC ACID. $C_7H_6O_5$.

Gallic acid, $C_6H_2(OH)_3COOH$, was first prepared by Scheele in 1786 by leaving an aqueous extract of gall nuts to stand in a warm place, and from time to time removing the layer of mould which formed on it; the crystalline precipitate which deposited from the solution was purified by recrystallization from water. Gallic acid, besides occurring in gall nuts, both free and in the form of its anhydride tannic acid, is also found free in sumach, divi-divi, the fruits of *Cæsalpinia coriaria*, in the leaves of *Arctostaphylos Uva-ursi*, and in tea and wine. It may be prepared from tannic acid by acid hydrolysis.

Gallic acid crystallizes in silken needles, and melts at 220°, forming pyrogallol and evolving carbon dioxide; it is sparingly soluble in cold water, but dissolves readily in hot water and in alkalis; alkaline solutions, like those of pyrogallol, absorb oxygen from the air, becoming brown in colour; they also reduce metallic solutions of silver or gold and Fehling's solution.

Gallic acid is converted into its anhydride digallic acid by heating with phosphorus oxychloride to 130° or by boiling with arsenic acid:—

$$\underset{\text{Gallic acid}}{2C_6H_2(OH)_3COOH} = \underset{\text{Digallic acid}}{C_{14}H_{10}O_9} + H_2O$$

This digallic acid precipitates gelatine from solution, and for

this reason it was regarded by Schiff* as being identical with natural gallotannic acid. This view was first shown by Walden† to be untenable, since the physical properties of the two substances are quite different, and the position was subsequently cleared up by the synthetic work of Fischer (see p. 213).

*Reactions.*

1. Gallic acid is precipitated from solution by lead acetate; on adding caustic potash a carmine-coloured precipitate is formed, which dissolves in excess to a raspberry-red solution.

2. Ferric chloride produces a blue-black colour or precipitate according to the strength of the solution; excess of ferric salt changes the colour to green, while excess of gallic acid reduces the ferric salt to ferrous and destroys the colour.

3. Iodine solution produces a transient red colour.

4. Gallic acid does not precipitate gelatine from solution. (Distinction from tannic acid.)

5. When heated with concentrated sulphuric acid it turns green and then purple, being converted into rufigallic acid, $C_{14}H_8O_8$, a substance used in dyeing.

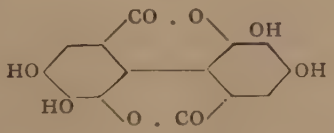
6. Potassium cyanide gives a pink colour which disappears on standing, but returns again on shaking with air.

7. Lime water gives a blue coloration or precipitate; in very dilute solutions a reddish colour is produced.

ELLAGIC ACID. $C_{14}H_6O_8$.

Closely related to gallic acid is the substance known as Ellagic acid, its name being obtained by the inversion of the word gallic.

Its constitution is, according to Graebe, best represented by the formula—




* Schiff: "Ber. deut. chem. Gesells.," 1871, 4, 232, 967; 1879, 12, 33; "Annalen," 1873, 170, 143.

† Walden: "Ber. deut. chem. Gesells.," 1897, 30, 3153; 1898, 31, 3167.

from which it will be seen that it may be considered to be produced by the abstraction of two molecules of water from two molecules of gallic acid, with simultaneous oxidation or removal of two atoms of hydrogen.

Synthetically it may be prepared by the oxidation of gallic acid by means of arsenic acid, or better by oxidizing gallic acid in acetic acid solution with potassium persulphate and sulphuric acid.*

Whether or not this substance occurs free in nature is not definitely established; certain it is, however, that ellagic acid can be readily obtained by the hydrolysis of ellagitannic acid, a substance which almost invariably accompanies gallotannic acid in the numerous vegetable products in which this latter occurs; it also occurs in conjunction with tannins of the pyrogallol class, and constitutes the bloom which is produced on leather by this type of tannin.

The most convenient natural sources are "divi-divi" (*Cæsalpinia coriaria*), "algarobilla" (*Cæsalpinia brevifolia*), "myrobalans" (*Terminalia Chebula*), etc. Aqueous extracts of these fruits on long standing frequently deposit ellagic acid, most probably by the action of a ferment contained in the plant; it is, however, prepared by pouring a hot concentrated alcoholic extract of divi-divi into cold water; the acid is thereby precipitated, and may be filtered and purified.

Ellagic acid is a yellow microcrystalline solid which is very slightly soluble in water, and therefore readily separates from aqueous solutions in which it is formed; it is also very slightly soluble in alcohol or ether, but dissolves somewhat readily in boiling pyridine. The dried substance treated with 1-2 drops of nitric acid gives, on dilution with 10-20 drops of water, a blood-red colour (Griessmayer's reaction).

Ellagic acid is used to some extent as a dye-stuff, being sold under the name of "Alizarine yellow in paste," for use with chromium mordants.

Catellagic, Metellagic, and Flavellagic acids are the names given by Perkin to artificially synthesized acids obtained by him. They are closely related to ellagic acid, but have not, as yet, been found to occur naturally.

* Perkin and Nierenstein: "J. Chem. Soc., Lond.," 1905, 87, 1415.

### THE CLASSIFICATION OF TANNINS.

With the present incomplete state of our knowledge concerning the chemical constitution of the tannins, it is difficult to make a proper chemical classification of these substances.

According to Trimble* the tannins may be divided into two main groups:—

1. Those containing about 52·2 per cent carbon.

   This group includes the tannins contained in oak galls (gallotannic acid), chestnut (wood and bark), sumach, pomegranate, etc.

2. Those containing about 59-60 per cent carbon.

   This group includes tannins of oak bark, kino, canaigre, ratanhia, catechu.

He points out that the fact of similar percentage composition would not itself be sufficient to justify this classification, but he finds that the classification still holds when the reactions towards certain reagents are compared as under:—

| | *Group* 1. | *Group* 2. |
|---|---|---|
| Ferric salts. | Blue colour and precipitate. | Green colour and precipitate. |
| Lime water. | White precipitate becoming blue. | Light pink precipitate becoming red and brown. |
| Bromine water. | No precipitate. | Yellow precipitate becoming brown. |

Dekker† proposes the following classification:—

1. Catechin tannins, occurring in gambier, catechu and *Hamamelis* bark.

2. True tannins—

(*a*) Gallic acid group . . . gallotannic acid; tea and sumach tannins.
(*b*) Ellagic acid group . . . divi-divi, algarobilla and myrobalan tannins.
(*c*) Oak bark group . . . the majority of red-producing tannins.

3. Pseudotannins (which do not form leather with hide), caffetannic acid and the tannins of maté, hops, etc.

Perhaps the best classification is the one given by Procter,‡ who divides tannins into two main groups:—

(A) *Pyrogallol tannins*, including divi-divi, galls, sumach,

* Trimble: *loc. cit.*, vol. ii. p. 132.
† Dekker: "De Looistoffen," Amsterdam, 1906.
‡ Procter: "The Principles of Leather Manufacture," London, 1903.

myrobalans, valonia, algarobilla, oak gall, oak wood and chestnut tannins.

These tannins have the following characteristics :—

1. They give with ferric salts a dark blue colour
2. They give no precipitate with bromine water.
3. They produce on leather a "bloom" consisting of ellagic acid.

(B) *Pyrocatechol tannins*, including all the pine barks, acacias, mimosas, oak barks (but not oak wood, fruits or galls), quebracho wood, cassia and mangrove barks, canaigre, cutch and gambier.

The tannins of this class are characterized by the following properties :—

1. They give with iron alum a greenish-black colour, though the reaction is liable to be rendered uncertain by the presence of other colouring matters.

2. When treated with bromine water, until the solution smells strongly of it, they give a yellowish or brown precipitate; in weak solutions the precipitate may form slowly.

3. The addition of concentrated sulphuric acid to a drop of the infusion produces a dark red or crimson ring at the junction of the two liquids; on dilution the liquid turns pink.

4. These tannins deposit no "bloom," but when boiled with acids deposit red insoluble colouring matters known as phlobaphenes (see p. 215).

Some of the tannins belonging to this group, notably gambier and cutch, contain phloroglucinol as one of their constituents; this substance may be tested for by moistening a pine wood shaving with a little of the infusion and then adding a little concentrated hydrochloric acid; the formation, after a short time, of a bright red or purple stain indicates the presence of phloroglucinol.

### TANNINS AS GLUCOSIDES.

Although many of the tannins are substances of a glucosidic nature and occur in the plant in combination with a carbohydrate complex such as glucose (e.g. gallotannic acid, p. 213) this has not as yet been established in all cases.

To determine whether a tannin is a glucoside or not the following procedure is recommended by Proctor.*

* Procter: "Leather Industries Laboratory Book, London," 2nd ed., 1908.

The Tannin must first be carefully purified from glucose, gums, or other bodies likely to interfere. This may be done by extracting according to Pelouze's method (p. 210), or, if the tannin is to be extracted from an aqueous solution, by agitating with ether to remove gallic acid and then saturating the aqueous solution with common salt and shaking with ethyl acetate, which extracts the tannin. The ethyl acetate is then evaporated off, the last traces being expelled by the repeated addition of small quantities of ether.

Another method is to extract with alcohol and to evaporate off the alcohol at as low a temperature as possible, and then to take up the residue with a large volume of water whereby the phlobaphenes (see p. 215) are precipitated and may be filtered off. The infusion is then precipitated with successive small quantities of lead acetate; the first and last portions are rejected and the middle fraction after washing is suspended in water and saturated with sulphuretted hydrogen. The precipitated lead sulphide is filtered off, and the solution is warmed to drive off excess of gas and then extracted with ethyl acetate.

Thus purified the tannin, or its washed lead salt, is heated to 100° for an hour or more in a sealed tube or boiled in a flask under a reflux condenser with hydrochloric acid (2 per cent). After cooling the mixture is allowed to stand for some time and is then filtered from any deposit which may have formed. The filtrate is shaken with ether to remove gallic acid and the aqueous solution boiled, neutralized with caustic soda and precipitated with basic lead acetate to remove any unchanged tannin or colouring matter; the solution is again filtered and any lead remaining in solution is removed by the addition of dilute sulphuric acid, excess of acid being carefully avoided. The solution is then neutralized and once more filtered and the clear filtrate heated to boiling with Fehling's solution when a red precipitate proves the presence of glucose.

### PROPERTIES AND DESCRIPTION OF INDIVIDUAL TANNINS.

As already stated the term Tannin is merely a generic name for the whole group of substances, though it has been, and still is, frequently used to mean a particular tannin, namely

that contained in oak galls. This substance is, however, better named gallotannic acid, as it is customary to name the tannins after the source from which they are obtained; thus quercitannic acid indicates the tannin of oak bark, sumac-tannin that derived from sumac, and so on.

## PYROGALLOL TANNINS.

### GALLOTANNIC ACID.

(Syn. Tannic acid, or merely "Tannin".)

The two chief commercial sources of gallotannic acid are:—

1. Turkish or Aleppo galls, produced by the gall wasp *Cynips gallæ*, which lays its eggs in the buds of *Quercus infectoria*. These contain from 50 to 60 per cent of gallotannic acid.

2. Chinese galls, produced by the burrowing of *Aphis chinensis* in the leaf-stalks of young twigs of *Rhus semialata*. These galls may contain up to 70 per cent of gallotannic acid.

Gallotannic also occurs in sumach (*Rhus Coriaria*), in tea, and in many other plants.

#### *Extraction of Gallotannic Acid.*

Gallotannic acid is best prepared by extracting finely-powdered gall nuts with a mixture of twelve parts of ether with three parts of alcohol; twelve parts of water are then added and, after shaking, the lower aqueous layer is run off from below and evaporated. The resulting tannic acid may be decolorized by boiling with animal charcoal.

Pelouze recommends the following method: The powdered material is heated under a reflux condenser with a mixture of thirty parts of ether, five parts of water, and two parts of alcohol. On cooling three layers of liquid are formed, of which the lowest contains 33 per cent, the middle 8 per cent, and the top 2 per cent of the tannic acid present in the substance.

Gallotannic acid forms an amorphous powder† which, when pure, is almost colourless; it is readily soluble in water, forming a solution with an astringent taste and which reacts

* What is known as "Crystal tannin" in commerce is not really crystalline; it is made by drawing a syrupy solution into threads and breaking these up after drying.

acid to litmus; it dissolves also in alcohol or glycerine, but is only sparingly soluble in ether and is insoluble in chloroform, benzene, ligroin or carbon disulphide; it is also insoluble in hydrochloric or sulphuric acids and is precipitated by these substances from its aqueous solutions; it is soluble in alkalis, and the solution, as in the case of gallic acid or of pyrogallol, rapidly absorbs oxygen from the air and darkens in colour.

When boiled with 2 per cent hydrochloric acid for some time, gallotannic acid is broken up into gallic acid.

If heated slowly from 160 to 215° and kept at the higher temperature for thirty minutes, carbon dioxide, water, pyrogallol and metagallic acid are produced. The pyrogallol volatilizes and condenses in the cooler part of the vessel.

The action of heat on tannins may also be studied by dissolving 1 gram of tannin in 5 c.c. of glycerine, heating slowly to 210° and maintaining the liquid at this temperature for half an hour. The liquid is then cooled and shaken with 20 c.c. of ether; after the addition of water the ethereal solution is separated and evaporated; the residue contains pyrogallol.

*Reactions.*

1. Ferrous sulphate, free from ferric salts, produces at first no change, but on exposure to air the solution darkens from oxidation.

2. Ferric chloride produces a blue-black colour or precipitate.

3. A dilute solution of iodine in potassium iodide gives a transient pink colour, as in the case of gallic acid.

4. Gallotannic acid is precipitated from solution by gelatine, and similarly combines with hide powder converting it into leather. (Distinction from gallic acid.)

5. Gallotannic acid precipitates proteins, alkaloids and many other organic substances from solution.

6. Lead nitrate or lead acetate gives precipitates of lead tannate. (*N.B.*—Neither pyrogallol nor gallic acid is precipitated by lead nitrate, though both give precipitates with lead acetate.)

7. Potassium cyanide gives a reddish-brown colour which changes to brown, but the red tint reappears on shaking with air.

8. Lime water gives a grey precipitate.

*Detection of Gallic Acid in Presence of Gallotannic Acid.* Gallic acid may be detected in the presence of gallotannic acid by dissolving the mixture in water and extracting the solution with ether; the ethereal extract on evaporation yields gallic acid which may be identified by the usual tests.

Gallotannic acid may also be separated from gallic acid by adding a solution of lead acetate strongly acidified with acetic acid; under these circumstances lead tannate is precipitated while lead gallate remains dissolved.

Similarly tannic acid is precipitated by many alkaloids and basic substances which have no action on gallic acid.

### THE CONSTITUTION OF GALLOTANNIC ACID.

The close relationship subsisting between gallotannic and gallic acids was first observed by Scheele, who, by allowing an infusion of gall nuts to undergo fermentation, obtained gallic acid. Within recent years this change has been studied anew by Fernbach,* who isolated a tannin splitting enzyme, tannase, from *Penicillium*, and also by Pottevin,† who isolated a similar enzyme from the mould *Aspergillus*.

This change, which may be represented by the equation—

$$C_{14}H_{10}O_9 + H_2O = 2C_7H_6O_5$$

may be effected more rapidly by boiling gallotannic acid with dilute sulphuric acid.

When, therefore, it was found by Schiff‡ that gallic acid could be converted back into the anhydride by means of phosphorus oxychloride it was assumed that this substance, which was called digallic acid, was identical with natural gallotannic acid or "tannin".

This view came to be generally accepted, although objections were raised from time to time on the ground that the physical constants, such as electrical conductivity and optical activity of natural tannin and synthetic digallic acid were different.§

Until 1912 there was considerable uncertainty as to whether tannin occurred in the plant combined with glucose in the

* Fernbach: "Compt. rend.," 1900, **131**, 1214.
† Pottevin: *id.*, 1900, **131**, 1215.
‡ Schiff: "Ber. deut. chem. Gesells.," 1871, **4**, 232.
§ Walden: *id.*, 1897, **30**, 3151; 1898, **31**, 3167.

form of a glucoside, or whether the sugar which is frequently found associated with it was merely an impurity.*

E. Fischer and Freudenberg,† on reinvestigating the question, found that "tannin," even after repeated careful purification, yielded about 7 to 8 per cent of glucose on hydrolysis with sulphuric acid; from this it was concluded that "tannin" or gallotannic acid as it occurs in nature is not identical with synthetic digallic acid, but is in reality a compound of five molecules of such a digallic acid with one molecule of glucose, in which the five hydroxyl groups of the sugar are esterified by five molecules of acid. Such a compound would be a pentadigalloyl glucose of the formula—

$$C_6H_7O_6[C_6H_2(OH)_3CO \cdot O \cdot C_6H_2(OH)_2 \cdot CO]_5 \text{ or } C_{76}H_{52}O_{46}$$

Actually two isomeric substances of this formula, with a molecular weight of 1700, have been synthesized. ‡

The one derived from meta-digallic acid, § i.e. penta- (*m*-digalloyl) *β*-glucose, has been found to be practically identical with Chinese tannin, and to differ from it only in regard to its specific rotation; this difference is, however, of no great significance considering the colloidal nature of the substance concerned.

Fischer, Bergmann, and Lipschitz have also synthesized a galloyl glucose of the formula—

$$C_6H_2(OH)_3 \cdot CO \cdot O \cdot CH \cdot (CHOH)_2 \cdot CH \cdot CHOH \cdot CH_2OH$$
$$\qquad\qquad\qquad

### ELLAGITANNIC ACID.

This tannin, which is commonly found together with gallotannic acid, is important as being the mother substance of ellagic acid, which is responsible for the bloom characteristic of pyrogallol tannins. The quantity of this substance present in different plants varies considerably; it is greatest in divi-divi. Amongst the other tannins giving ellagic acid bloom may be mentioned algarobilla, myrobalans, chestnut tannin, pomegranate tannin, valonia, etc.

Ellagitannic acid, unlike ellagic acid (p. 205), is soluble in water or alcohol; prolonged boiling with water converts it into ellagic acid. It has been variously described by different authors as a glucoside, as a hydrated soluble form of ellagic acid, or as a condensation product of ellagic acid with gallic acid.*

## PYROCATECHOL TANNINS.

### CATECHU TANNIC ACID.

Catechu tannic acid is the name given to the tannin contained in gambier catechu and in Bombay catechu or cutch,† a substance obtained by evaporating an aqueous extract of the bark of various trees (see below). A similar tannin is also contained in kino.

Little is known as to its constitution, but it is believed to be an anhydride of catechin.

### CATECHIN.

This substance, which is obtained from *Acacia catechu*, *Ouroparia catechu*, mahogany wood, *Mimosa*, and pyrocatechol tannins in general, is not in itself a tannin since it does not precipitate gelatine, but it is converted into a tannin, namely catechu tannic acid, by loss of water, a change which may be rapidly brought about by heating to 120° or above.

Catechin may be prepared by extracting powdered catechu with ether; the crude material obtained on evaporating off the ether may be purified by crystallization from water.

* Cf. Nierenstein: "Ber. deut. chem. Gesells.," 1907, 40, 4575; 1909, 42, 353; 1910, 43, 1257.

† This substance is used largely for dyeing.

Catechin forms colourless glistening needles, which, when dry, melt at 175-177°. It is readily soluble in alcohol and ethyl acetate, not so readily soluble in ether, and only slightly soluble in cold water.

With ferric chloride alone it gives a green colour, but with ferric chloride and sodium acetate a dark violet.

It gives the phloroglucin reaction with pine wood shaving and hydrochloric acid.

Potash fusion gives protocatechuic acid and phloroglucinol.

### QUERCITANNIC ACID.

Quercitannic acid is the name given to the tannin of oak bark, which is not identical with the tannin of oak galls.

Pure quercitannic acid yields no glucose on hydrolysis, though levulose is nearly always present in oak bark.

Although much work has been done on the oak bark tannins by various workers, notably Etti, Böttinger and Löwe, nothing definite is known as yet regarding their constitution.

Procter summarizes the present state of our knowledge by saying that, on the whole, it seems probable that the principal tannin of oak bark is a purely catechol tannin, and that the gallic and ellagic acids which have been detected in it are due to an admixture of the gallotannic and ellagitannic acids present in oak wood.

A great many more tannins are known, but too little is known about their composition to justify their inclusion here.

### PHLOBAPHENES.

Among the products of the decomposition of tannins by boiling with acids must be mentioned the substances known as Phlobaphenes. The name derived from the Greek (φλοιός—bark, and βαφή—dyeing) was first given by Stahelen and Hoffstetter,* in 1844, to a red-brown substance obtained by them by adding water to an alcoholic extract of bark which had previously been extracted with ether to remove fats or waxes. It has since been shown that aqueous extracts of bark containing tannin, deposit from solution a substance known as oak-red or phlobaphene, and that this substance is more rapidly

* Stahelen and Hoffstetter: "Annalen," 1844, 51, 63.

produced by warming concentrated solutions of tannin with sulphuric acid.

Inasmuch as phlobaphenes are produced by any process which tends to remove water, such as heating tannins to a high temperature or prolonged boiling or heating under pressure, they are regarded as anhydrides of the tannins; besides being thus produced artifically, they occur also in nature side by side with the tannins from which they can be produced.

They are red-coloured substances and are practically insoluble in water though they dissolve in solutions containing tannic acid; also they dissolve in alcohol and in alkaline solutions.

The formation of phlobaphenes by treatment of a tannin with acid is characteristic of pyrocatechol tannins (p. 208) in just the same way as ellagic acid is produced from pyrogallol tannins.

A number of different phlobaphenes are known, such as kino-red, catechu-red, oak-bark red, etc.

### PHYSIOLOGICAL SIGNIFICANCE OF TANNINS.

It is manifestly a difficult matter to ascertain the significance of tannins in the life of the plant, more especially as these substances vary in different species, so that what may be true for one is not necessarily true for all.

It is, therefore, not surprising to find that several ideas have been put forward.

With regard to the origin of tannins practically nothing of fundamental importance is known.

According to the investigations of Kraus, tannin, although not a direct photosynthetic product—as is indicated by the fact that the tannin does not increase in the leaves of plants which are able to photosynthesize in dull light—is not formed unless carbon dioxide and light are available. He found that etiolated leaves produced no tannin, and that the amount of this substance in shaded leaves was less than that contained in the leaves of the same plant fully exposed to the sun. The tannin thus formed is translocated to the stem and root.

Similarly Dekker* finds that light is requisite for the formation of tannin, and that the tannin content of leaves

* Dekker; "Rec. trav. bot. néerlandais," 1917, 14, 1.

considerably decreases in darkness owing to translocation and other processes.

This, however, is not the only origin for tannin, for if tannin-containing seeds, e.g. the oak, be germinated in darkness, there is an increase in the amount of tannin; further, the production of various aromatic compounds may be a stage in the synthesis of proteins, and some of these may eventually give rise to tannin.

The facts regarding the distribution of tannin have an important bearing on the subject. It is abundant in leaves; in parts in which growth is very active, such as growing points; in galls and other pathological growths; also it is found in association with secretory organs, such as gland cells of *Sarracenia* and *Utricularia*, and in parts in which the protoplasm is especially irritable, such as pulvini. Pfeffer found that in young fully formed pulvini no tannin occurs, but it appears soon after movements commence and gradually increases in quantity until the leaf dies.

In the case of *Robinia pseudacacia* the pulvini of the leaflets contain less tannin than the main pulvinus, which is much less sensitive than are the secondary pulvini.

The consideration of these facts supports the conclusion arrived at by Sachs that tannin results from intense metabolism such as occurs in active leaves; in rapid tissue formation, as in galls and vegetative apices; during germination and secretion; and as a consequence of particular stimulation, as in mobile pulvini.

Various facts on the relation between tannin and other substances such as starch, sugar, resin, etc., have led to various opinions.

That starch frequently is contained in the same cells with tannin suggests a connexion between the two, and it is not impossible that the starch may contribute the glucose for the construction of the tannin. In the case of *Pinus*, it has already been mentioned that in the spring, when the flow of resin is most copious, the tannin decreases as the resin increases; also the cells surrounding the epithelium of resin ducts contain tannin and starch. Wiesner, therefore, concluded that tannin is an intermediate product in resin formation.

Tannin is not uncommon in unripe fruits, and the amount of these astringent substances diminishes during ripening.

According to Bassett* "the amount of tannin in fruits varies with certain factors, such as injury, length of time between removal from tree and analysis, etc. The presence and relative amount of this tannin or tannin-like body is controlled by the presence of certain enzymes which vary in amount and activity during the growth of these fruits."

Buignet, from the fact of the diminution of tannin and starch which occurs concurrently with the increase in sugar, considered that the sugar in the ripe fruit (e.g. *Musa*) is formed from these two substances. This opinion, however, is not held by Gerber who investigated the same phenomenon. In *Diospyros Kaki* he found the young fruit to be very astringent, but not so the ripe fruit. He considers that the tannins disappear by complete oxidation without the formation of carbohydrates. One reason for his opinion is that in the conversion of tannin into carbohydrate more carbon dioxide would have to be liberated than oxygen absorbed, whereas in fruits the relation is the reverse.

On the other hand, he does consider the tannins to be of some value, for they, by the formation of pectins, may limit the loss of carbohydrate.

Further, inasmuch as the pleasing odours of fruit are acquired after the tannin has disappeared it is not impossible that the latter may have some connexion with the formation of fruit esters.

Other suggestions regarding the value of tannin are not wanting; thus Moore† states that it may play an important part in the lignification of cell walls.

More recently Drabble and Nierenstein‡ have come to the conclusion that tannins play an important part in cork formation, and are acted upon in the plant by formaldehyde and acids and are precipitated in the walls of the cork cells. Reasons for this theory may be alluded to briefly.

* Quoted from the footnote appended to a paper on the Toxicity of Tannin by Cook and Taubenhaus: "Delaware Coll. Agric. Exp. Station," Bull., 91, 1911.

† Moore: *loc. cit.*

‡ Drabble and Nierenstein: "Biochem. Journ.," 1906, 2, 96.

There occur in plant tissues tannins; phenols such as phloroglucinol, resorcinol and hydroquinone; and hydroxybenzoic acids, such as gallic, salicylic, and protocatechuic acids.

When these substances are treated with hydrochloric acid and formaldehyde various condensation products are precipitated. These condensation products can be produced from gallic acid, pyrogallol, protocatechuic acid, phloroglucinol, salicin, tannic acid, and other substances, simply by passing a slow stream of carbon dioxide through the mixture of formaldehyde and the tannic acid, for example.

The reactions given by these bodies are similar to those characteristic of cork; thus they are insoluble in Schweitzer's reagent and strong sulphuric acid, but readily dissolve in strong potash. It is, therefore, possible that in cork formation similar condensation products may play a part, for the requisite materials are present in the plant.

Further, in the plants examined, the presence of gallic or tannic acids was indicated in the immediate neighbourhood where cork was being formed, and by suitable means there can be obtained from cork, products having the same mother substance as the condensation products mentioned above.

Still more recently Van Wisselingh has published certain observations from which he concludes that tannin plays an important part in the formation of cell walls in certain cases, for instance *Spirogyra*. He does not consider it a reserve food-material as such, but rather a soluble substance which the plant makes use of in elaborating other materials. This conclusion is in agreement with the opinions held by Wingand and published in 1862. Van Wisselingh worked with *Spirogyra*, and the main facts on which he based his conclusions are as follows. Cells which are about to conjugate are rich in tannin, and as the process of conjugation proceeds, there is a gradual diminution in the amount of this substance, so that the mature zygospore contains nothing more than mere traces.

If conjugation be interrupted at an early stage, there is still an increase of tannin, so that when death results there is relatively a large quantity present. This accumulation may be used as an argument in support of the view that tannin is a waste product. Van Wisselingh, however, remarks that this should not be a source of wonder, for in this case "It is not

the intention of Nature that it should be wasted. Nature ensures a sufficient supply of tannin in *Spirogyra*, because this substance is required in development, as for instance in conjugation and spore-formation. The occasional failure to conjugate as a result of which much tannin is lost, does not prove that it is a waste product and not a plastic material."

The author in question also found that a diminution of tannin occurred during the formation of the cell wall after nuclear division, and if the tannin were precipitated during the earliest phases of cell division, the cell wall was not formed although the nucleus divided into two quite normally. *Cladophora*, which does not contain tannin, was used as a control; it was found that by keeping the filament in a solution of antipyrine, the reagent used in the experiment on *Spirogyra*, the cell-wall formation was not disturbed.

It must be mentioned that Van Wisselingh does not claim that tannin is the only substance used in cell-wall formation, nor does he maintain that the only physiological significance of tannin is its use as a plastic material.

Finally, in this particular connexion, it may be mentioned that tannin may play a part in the formation of various pigments such as anthocyan and erythrophyll, for similar decomposition products (compounds allied to the phenols) may be obtained from each.

The fact that some Fungi can make use of tannin as a food material provided that it is not in excess, and the facts that many are glucosides, and that oxidation readily takes place with the ultimate formation of oxalic acid and carbon dioxide, suggest that the substances under consideration may be reserve food-material.

Thus Schell, while acknowledging that tannin may sometimes be a bye-product of metabolism, considered that at other times it might be used up in the construction of higher compounds which would serve as food. He found that, in the germination of the oil-containing seeds of *Echium vulgare* and other Boraginaceae, as the oil is used up the tannin begins to play a part in the constructive metabolism and gradually diminishes in amount. Further, if such seeds be germinated in the light the tannin increases in quantity. For these and other reasons he concluded that such a use of tannin only ob-

tained when there was a scarcity of the more normal foods such as starch and oil.

A consideration, however, of other facts does not tend to support the idea of tannin being of the nature of a reserve food. Hillhouse,* for example, found that if a fuchsia having an abundant supply of tannin be grown in the dark, there is no diminution in the substance in question. Then again the facts of its distribution are against this particular view; for example, it does not occur in sieve tubes which transport both sugar and other food substances; there is, in many cases, not a great discrepancy in the tannin-content of fully mature and fallen leaves, for naturally it would be expected that if tannin were of any considerable value as a food-stuff it would not be accumulated in bark and old leaves but would be translocated out of such places before they were cast off, the same as are other materials in the generality of cases. But against this argument may be cited the fact that fallen leaves may contain substances of undoubted value to the plant, such as nitrogen and phosphorus, and even glucose and starch. In evergreen leaves there is no diminution in the quantity of tannin during the winter months, which may mean that either it is of no great value or that, since growth is more or less at a standstill, the plant has more food than it requires immediately, or that it subserves some biological function; thus Warming has suggested that in this particular connexion the tannin may be of value in protecting the plant against undue evaporation during the winter, and further it may be a means of rapidly restoring lost turgor.

On the other hand the figures obtained by Levi and Wilmer, mentioned above, require some explanation; why should a minimum of tannin occur in the leaves in June when photosynthesis is so very active? is it used up in the construction of other substances or is it merely translocated to other parts such as the bark? If the latter be true, the further question arises, then why should it be transferred at one time of the year and not at another?

Of course, it is possible that these and like variations may be explained by the varying conditions of, say, light, temperature and moisture; and with regard to this variation in the

* Hillhouse: "Midland Naturalist," 1887-8.

amount of tannin, more especially in germinating seeds, van Wisselingh points out that the amount found at any particular moment represents the balance as it were of the tannin account; that is to say, if more tannin is formed than is decomposed, an increase in the tannin content will result and vice versa, so that in one and the same plant there will be sometimes an increase and sometimes a decrease according to the conditions obtaining. It does not necessarily follow, and this is applicable to many things besides tannin, that because there is an increase in the amount, therefore the substance is of no value in constructive metabolism.

Tannin has been considered an important constituent of the osmotic substances of the cell; although this may be true for some tannins it probably does not hold for all, since no ill effects follow the precipitation of tannins in the living cell by means of methylene blue; also, in certain cases, it is not renewed when precipitated.

A biological significance is not infrequently attached to tannins; thus it may be of use against animals, it may be connected with the activity of nectaries in providing sugar, and it has been suggested by Moore that when it occurs in the epidermis of leaves, it may play a part in the opening and closing of stomata.

Finally, it may be of considerable value as an antiseptic, preventing the germination and growth of parasitic Fungi. In this connexion Cook and Taubenhaus* have found that in many cases tannin has a tendency to retard or inhibit the growth of Fungi, the parasitic forms being more sensitive than the saprophytic. In some cases the spores are killed, whilst in others germination is much impeded. On the other hand, low percentages of tannin may in some instances stimulate germination and also fruiting. The behaviour of Fungi towards tannin varies with the species and sometimes even with the individual, more especially in the case of spores.

To conclude, the different substances included under the term Tannin are so numerous as to make it improbable that they all have the same physiological significance.

*Cook and Taubenhaus: "Delaware Coll. Agric. Exper. Station" Bull., 91, 1911.

# SECTION VI.

## PIGMENTS.

### CHLOROPHYLL.

As is well known, chlorophyll is contained in the chloroplasts which are universally present in green plants and vary considerably in their size, shape, and number within the cell. With regard to their structure there has been much dispute. It is, however, generally agreed that the structure of the plastids is either reticulate or vacuolate.

The pigment itself is variously stated to be dissolved in some oily substance which is held in the channels and meshes of the plastids, or to exist in the form of a precipitate; and with regard to the distribution of the pigment within the plastid there is again some dispute. According to many, it is distributed evenly throughout the stroma, whilst, on the other hand, others maintain that it is restricted to the peripheral layers of the plastid.

Amongst the most recent contributions to the subject is the investigation of Priestley and Irving * on the chloroplasts of certain species of *Selaginella* and *Chlorophytum*. They find that the pigment is restricted to the peripheral regions of the chloroplast, where it is held in the meshes of the network of the matrix. They agree with Timiriazeff's views that the function of the chlorophyll necessitates its distribution in very thin layers in order that the amount of energy set free may be as great as possible.

With regard to the origin of the chloroplast there is also some dispute. The general view, due originally to Schimper and Meyer, appears to be that plastids do not arise *de novo* within the cell, but by the division of pre-existing plastids, so that, in this respect, there is continuity between parent and offspring. This has led to the conception that originally the

* Priestley and Irving: "Ann. Bot.," 1907, 21, 407.

chloroplasts once had a separate individuality, and that, in a sense, ordinary plants are parasitic upon the imprisoned plastids which have become permanent members of the structures of the cell.

On the other hand, other investigators hold that the chloroplasts may arise from differentiated parts of the protoplasm, which parts are not plastids. Lewitski * draws attention to the presence of minute bodies occurring in the protoplasm, but not in the nucleus, which he calls mitochondria, chondriosomes, etc. These structures, which he considers are essential parts of the cytoplasm, increase by division, and give origin to the plastids. For instance in the pea, *Pisum sativum*, and the asparagus, *Asparagus officinale*, the mitochondria of the cells of the stem apex give rise to chloroplasts, whilst those of the apex of the root are converted into leucoplasts. Meyer,† however, is opposed to these conclusions. Miller‡ finds that very minute chloroplasts occur in the cotyledons of the seed of *Helianthus annuus;* they increase in size and divide by fission as germination proceeds and maturity is reached.

Mottier§ agrees that some forms of chondriosomes give origin to chloroplasts and leucoplasts. He considers them to be permanent structures of the cell, and that certain kinds function as transmitters of hereditary characters.

In green plants chlorophyll may occur in places where light seemingly cannot penetrate, at any rate in any quantity, for instance, in the cortex internal to the periderm—not only in small twigs, but also of larger branches—in the medullary rays and even in the pith.‖ Also it may occur in the cotyledons of seeds before they are set free from the ovary or from the cone; *Pinus*, *Euonymus europœus*, and species of *Cucurbita* are familiar examples. In some of these cases light no doubt does penetrate through the walls of the superposed cells;

* Lewitski: "Ber. deut. bot. Gesells.," 1910, **28**, 538.

† Meyer: *id.*, 1911, **29**, 158. See also Schmidt: "Prog. Rei. Bot.," 1912, **4**, 163; Forenbacher: "Ber. deut. bot. Gesells.," 1911, **29**, 648; Woycicki: "Sitz. Warschauer Ges. Wiss.," 1912, **5**, 167; and Löwschin: "Ber deut. bot. Gesells.," 1913, **31**, 203.

‡ Miller: "Ann. Bot.," 1910, **24**, 693. A *résumé* of the literature is given by Cavers in "New Phyt.," 1914, **13**, 96, 170.

§ Mottier: "Ann. Bot.," 1918, **32**, 191.

‖ See Scott: *id.*, 1907, **21**, 437.

this may be well seen if the seeds be removed and the lumen of the fruit of the vegetable marrow be cleaned out. It is hardly necessary to remark that if the chlorophyll in these deeply-seated tissues be functional, its contributions to the food-stuffs of the plant, as Goldflus * has pointed out, must be of considerable value.

But in some cases the pigments of such chloroplasts may not be the same as those of the ordinary chloroplasts of the leaf; thus, according to Monteverde and Lubimenko,† the seeds of many Cucurbitaceæ contain not chlorophyll, as ordinarily understood, but chlorophyllogen, which may pass over into chlorophyll under the influence of light and some other factor, possibly enzymic.

Also it must be remembered that it does not follow that because chlorophyll is present, photosynthesis necessarily takes place, even though the requisite conditions, light and supply of raw material, obtain. Thus it appears probable that the chlorophyll in green parasites is not functional, and the same holds for the chlorophyll in the gynæcium of certain plants, e.g. *Ornithogalum arabicum*. At any rate, in these cases the photosynthetic power is so small as to be masked by the respiratory activity.

Attention may here be drawn to the work of d'Arbamont,‡ who considers that the plastids containing chlorophyll may be divided into two classes, chloroplasts and pseudochloroplasts. Of these the former include those bodies usually termed chloroplasts, and are characterized by the fact that they do not swell in water, and do not, as a rule, stain when treated with acid aniline blue. On the other hand, pseudochloroplasts swell in water and do stain with aniline blue. In some cases plants may contain pseudochloroplasts only.§

With regard to the conditions necessary for the formation of chlorophyll, light is the most important, but in addition a certain degree of temperature, as well as the presence of certain substances, such as iron and magnesium, are essential. There

* Goldflus: "Rev. Gén. Bot.," 1901, **13**, 49.

† Monteverde and Lubimenko: "Bull. Jard. Imp. Bot., St. Pétersbourg," 1909, **9**, 27.

‡ D'Arbamont: "Ann. Sci. Nat. Bot.," 1909, **9**, 197.

§ See Belzung: *id.*, 1891, **13**, 17; "Journ. Bot.," 1895, **9**, 67, 102.

is, however, some dispute regarding other factors. Palladin * states that chlorophyll formation is an oxidative process, and, as a result of his experiments, finds that etiolated leaves on exposure to daylight will not form chlorophyll unless a supply of carbohydrate is available. If an etiolated leaf does not contain carbohydrate, then greening will take place if the cut leaf be placed in a solution of sugar. Almost any sugar will do, e.g. sucrose, maltose, glucose, fructose, or raffinose; success was also obtained by the use of glycerine. The solution used must be neither too weak nor too strong; a strong solution of sucrose, for instance, will retard the chlorophyll formation because it will depress oxidative processes. On the other hand, Issatchenko † finds that etiolated leaves of certain plants, e.g. those of *Vicia Faba*, when detached from the plant and placed in strong sugar solution, even 50 per cent, will form chlorophyll. He considers that light is the all-important factor.

With regard to the substances which immediately precede chlorophyll, and from which chlorophyll is formed, nothing definite is known.

The chemical study of chlorophyll dates from the year 1819, when Pelletier and Caventou ‡ first applied this name to the green leaf pigment without, however, isolating the substance. Since then, numerous workers have attempted to prepare chlorophyll in a pure condition, but the methods employed in most cases were of too drastic a nature for the substance to escape destruction. Previous to 1911, there was no chemical evidence to show that chlorophyll was not a single chemical individual, although Stokes,§ Sorby,‖ and others had obtained spectroscopic evidence pointing to the existence of more than one substance; confirmatory evidence was subsequently obtained by Tswett.¶ In 1912, however, Willstätter and Isler ** definitely showed that chlorophyll as

* Palladin: "Ber. deut bot. Gesells.," 1891, **9**, 194, 229; 1902, **20**, 224; "Rev. Gén. Bot.," 1897, **9**, 385.

† Issatchenko: "Bull. Jard. Imp. Bot., St. Pétersbourg," 1906, **6**, 20.

‡ Pelletier and Caventou: "Ann. Chim. Phys.," 1819, **9**, 194.

§ Stokes: "Proc. Roy. Soc.," 1864, **13**, 144.

‖ Sorby: *id.*, 1872, **21**, 442.

¶ Tswett: "Ber. deut. bot. Gesells.," 1906, **24**, 326; 1907, **25**, 137; "Ber. deut. chem. Gesells.," 1908, **41**, 1352

** Willstätter and Isler: "Annalen," 1912, **390**, 269.

ordinarily obtained, and to which they had originally assigned the formula $C_{55}H_{72}O_6N_4Mg$, is in reality a mixture of two substances :—

Chlorophyll *a* $C_{55}H_{72}O_5M_4Mg$ *
and Chlorophyll *b* $C_{55}H_{70}O_6N_4Mg$.

Accompanying chlorophyll are three yellow or reddish-brown pigments, Carotin, Xanthophyll, and Fucoxanthin (the latter occurring only in brown algæ), which are known collectively as the Carotinoids. Owing to the similarity in solubilities between these substances and chlorophyll, their complete separation is a matter of some difficulty; it was first effected by Willstätter and Hug.†

The average proportions in which these various constituents occur in different plants have been determined by Willstätter, and are approximately as follows :—

| | In Land Plants.‡ | Brown Algæ ‡ (*Fucus*). | Green Algæ ‡ (*Ulva*). |
|---|---|---|---|
| Chlorophyll *a* . . . | ·62 | ·16 | ·093 |
| ,, *b* . . | ·22 | ·01 | ·066 |
| Carotin . . . . | ·055 | ·0312 | ·014 |
| Xanthophyll . . . | ·093 | ·0305 | ·036 |
| Fucoxanthin . . . | — | ·059 | — |

From these figures the following interesting deductions may be made :—

1. The molecular proportions between chlorophylls and carotinoids are as 3·5 to 1 ‡ in terrestrial plants, but only 1 to 1 in the case of algæ.

2. In the brown algæ chlorophyll *a* predominates, only about 5 per cent of the mixture being chlorophyll *b*; in terrestrial plants, on the other hand, the proportion is pretty constantly about 3 : 1.

3. In the green algæ there is relatively more of chlorophyll *b*.

Concerning the physiological significance of these sub-

* For the physical characteristics of these two substances see page 231.

† Willstätter and Hug : "Annalen," 1911, 380, 177.

‡ These figures are percentages calculated on the dry material.

§ With regard to this ratio, it has been stated by Willstätter that it is remarkably constant, and that there is a greater variation between different leaves of the same plant than between corresponding leaves of different plants. This view is, however, contested by Borowska and Marchlewski ("Biochem. Zeitschr.," 1913, 57, 423), who hold that it is entirely dependent on external circumstances, such as soil, stage of growth, etc.

stances it has been suggested by Willstätter* that since chlorophyll *b* ($C_{55}H_{70}O_6N_4Mg$) contains more oxygen than chlorophyll *a* ($C_{55}H_{72}O_5N_4Mg$), the former compound is produced by the action of chlorophyll *a* upon carbon dioxide during assimilation, and that chlorophyll *b* is then reconverted into chlorophyll *a* with evolution of oxygen. On the other hand, the molecular formulæ of carotin ($C_{40}H_{56}$) and xanthophyll ($C_{40}H_{56}O_2$) only differ by two atoms of oxygen, and the close association between the carotinoids and chlorophyll may be explained by assuming that the function of carotin is to reduce chlorophyll *b* to chlorophyll *a*, being itself oxidized to xanthophyll, and that the latter compound is reconverted by some enzyme into carotin with evolution of oxygen.

Quantitative measurements of the relation between the amount of carbon dioxide assimilated and the weight of chlorophyll concerned have been made by Willstätter and Stoll.† A regular stream of air containing a known amount of carbon dioxide was passed over from 5 to 20 grams of leaves contained in a small illuminated glass vessel immersed in a constant temperature water-bath. By estimating the amount of carbon dioxide in the issuing gas and the amount of chlorophyll in the leaves, they determined the so-called assimilation number for different leaves which was the ratio between the amount of carbon dioxide assimilated per hour and the weight of chlorophyll concerned in the assimilation. Experiments with normal, autumnal, and etiolated leaves showed that the assimilation is not always proportional to the chlorophyll content, which may be explained by assuming that some enzyme takes part in the process. The fact that in leaves rich in chlorophyll increased illumination produces no increased assimilation, whereas a rise in temperature does, is attributed to the accelerating effect of increased temperature upon enzyme action. In the case of leaves deficient in chlorophyll, on the other hand, increase of temperature has but little effect, whereas such leaves are very susceptible to increased illumination. The explanation in this case is that there is more than sufficient enzyme for the chlorophyll, but that the greatest assimilative effect can only be attained when all the chlorophyll is exerting its maximum

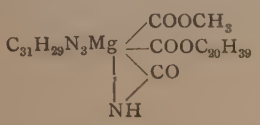
* Willstätter: "Untersuchungen über Chlorophyll," p. 237.

† Willstätter and Stoll: "Ber. deut. chem. Gesells.," 1915, 48, 1540.

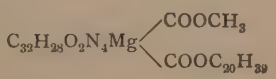
activity. Attempts to bring about assimilation with chlorophyll outside the leaf failed, presumably owing to the absence of this enzyme. The removal of epidermis from the under surface of leaves had no deterrent effect on assimilation, but a slight pressure applied to the leaves brought assimilation to a complete standstill.

## THE CONSTITUTION OF CHLOROPHYLL.

As already stated, chlorophyll was first isolated from its accompanying yellow pigments, the carotinoids, by Willstätter and Hug in 1911, and in the following year it was shown by Willstätter and Isler that the chlorophyll so obtained was not a single substance, but a mixture of two distinct substances, chlorophyll *a* and chlorophyll *b*, in the proportion roughly of three molecules of the former to one of the latter. The constitutions provisionally assigned to these two substances are given by the following formulæ :—




Chlorophyll *a*




Chlorophyll *b*

from which it may be seen that they are both esters of methyl and phytyl alcohol ($C_{20}H_{39}OH$), and that the former contains what is known as a lactam grouping.

The recognition of magnesium as an essential constituent of chlorophyll, which is due to Willstätter,* has proved of immense value in the study of the degradation products of chlorophyll.

By the action of alkalies and acids respectively upon the two chlorophylls, it has been found possible to divide the degradation products of chlorophyll into two groups :—

1. Those that retain magnesium, known as *Phyllins.*

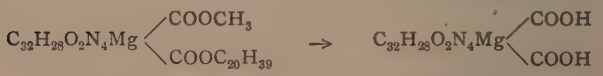
2. Those that are free from magnesium, known as *Porphyrins.*

* Willstätter : "Annalen," 1906, 350, 48.

*The Action of Alkalies.*

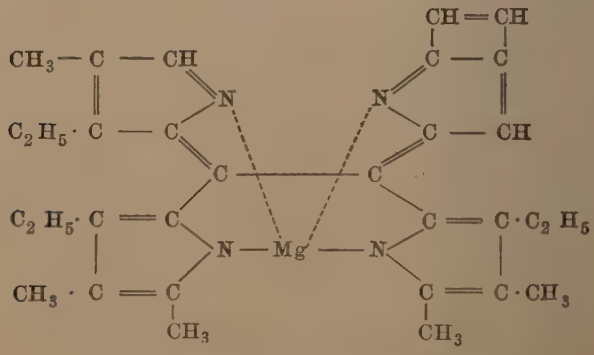
When the two chlorophylls are treated with the calculated amount of concentrated methyl alcoholic potash their ester groups are hydrolysed, and two isomeric tribasic acids result from each which are known as chlorophyllin and isochlorophyllin *a* or *b*, as the case may be:—

Chlorophyll *a* — Chlorophyllin *a* and Isochlorophyllin *a*




Chlorophyll *b* — Chlorophyllin *b* and Isochlorophyllin *b*

Chlorophyllin *a* when heated with alkali loses carbon-dioxide, and yields two isomeric dibasic acids, glaucophyllin and rhodophyllin, $C_{31}H_{32}N_4Mg(COOH)_2$, and at a higher temperature it loses two molecules of carbon dioxide, yielding a monocarboxylic acid, pyrrophyllin, $C_{31}H_{33}N_4Mg(COOH)$. By heating with soda lime the third molecule of carbon dioxide may be removed with the formation of aetiophyllin a substance containing no carboxyl group at all, and to which the following formula is assigned:—

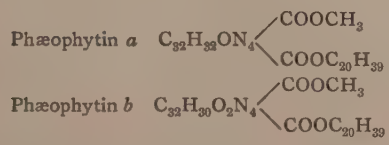



Aetiophyllin $C_{31}H_{34}N_4Mg$

*The Action of Acids.*

Acids, especially oxalic acid, remove magnesium from all derivatives containing this element, replacing it by two atoms of hydrogen without altering the rest of the molecule.

Thus chlorophyll *a* and *b* give by removal of Mg the compounds




respectively, while chlorophyllin *a* gives phytochlorin *f* and *g*, $C_{32}H_{32}ON_4(COOH)_2$. On the other hand, glauco and rhodophyllin by removal of magnesium give glauco and rhodoporphyrin $C_{31}H_{34}N_4(COOH)_2$, while pyrrophyllin yields pyrroporphyrin $C_{31}H_{35}N_4(COOH)$. By removing the last carboxyl from the latter compound a substance aetioporphyrin $C_{31}H_{36}N_4$ is obtained, which is the magnesium free analogue of aetiophyllin $C_{31}H_{34}N_4Mg$.

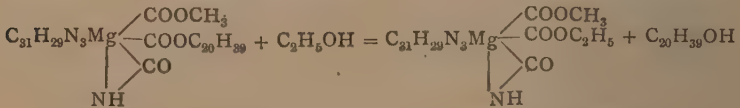
## CRYSTALLINE AND AMORPHOUS CHLOROPHYLL.

The physical constants of these substances as determined by Willstätter and his pupils are as follows:—

| *Chlorophyll (a and b).* | *Chlorophyll a.* $C_{55}H_{72}O_5N_4Mg$. | *Chlorophyll b.* $C_{55}H_{70}O_6N_4Mg$. |
|---|---|---|
| Analysis agrees with formula $C_{55}H_{72}O_6N_4Mg$. | | |
| Bluish-black glistening powder, with metallic lustre. | Bluish-black powder. | Dark green or greenish-black glistening powder. |
| Appears crystalline under the microscope. | Bluish-black powder. | Dark green or greenish-black glistening powder. |
| No definite M.P. | Sinters and forms a viscous mass at 117-121° | Sinters at 86-92°, and becomes viscous at 120-130°. |
| Practically insoluble in cold light petroleum, but dissolves readily on addition of a few drops of methyl or ethyl alcohol. | Very sparingly soluble in light petroleum, but dissolves very easily in most organic solvents. | Quite insoluble in light petroleum, and is generally somewhat less soluble than chlorophyll *a*. |

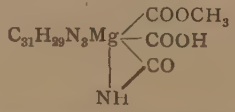
| *Phase Test.* | *Phase Test.* | *Phase Test.* |
|---|---|---|
| (i.e. hydrolysis in ethereal solution, with methyl alcoholic potash), gives a transient brown coloration (cf. p. 238). | Transient pure yellow colour. | Transient brilliant red colour. |

From the above data it will be seen that neither ordinary chlorophyll (*a* and *b*) nor either of the constituents of this mixture show any marked tendency to crystallize which at first sight would appear to be in contradiction with the well-known fact first observed by Borodin * that when green leaves are moistened with alcohol, and allowed to evaporate slowly under a coverslip, crystals of chlorophyll may be observed under the microscope. Willstätter and Benz † described a method of obtaining this substance in quantity from *Galeopsis tetrahit*, and later Willstätter and Stoll ‡ showed that this so-called crystalline chlorophyll was not present as such in the plant, but was a secondary product produced by the action of the alcohol upon the chlorophyll under the action of an enzyme chlorophyllase. The phytyl group is thereby replaced by the ethyl group as illustrated by the equation :—



Amorphous Chlorophyll *a* — Crystalline Chlorophyll *a*

For the monomethyl ester of chlorophyllin *a* Willstätter has proposed the name chlorophyllide *a*,



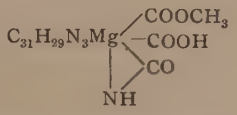
and adopting this nomenclature, amorphous chlorophyll would be termed phytylchlorophyllide, while crystalline chlorophyll would be ethylchlorophyllide.

Chlorophyllase belongs to the same class of enzymes as lipase ; the latter substance, however, is only able to hydrolyse amorphous chlorophyll, replacing the phytoxyl group by hydroxyl ; it cannot effect alcoholysis.

* Borodin : " Bot. Ztg.," 1882, **40**, 608.
† Willstätter and Benz : " Annalen," 1907, **358**, 267.
‡ Willstätter and Stoll : *id.*, 1910, **378**, 18.

On the other hand, working with methyl alcohol and chlorophyllase, it has been found possible to replace the phytyl group by methyl, forming methylchlorophyllide, which is the methyl analogue of ethylchlorophyllide or crystalline chlorophyll; it is best obtained by treating fresh leaves with 50-60 per cent methyl alcohol; if prepared from *Heracleum* it is sparingly soluble in ether and crystallizes from that solvent in steel-blue glistening prisms; that prepared from stinging nettles is slightly less soluble in ether and crystallizes in triangular and hexagonal plates.

By acting in moist ethereal solution in the absence of alcohol, ordinary hydrolysis was effected with the formation of the monomethyl ester of the chlorophyllin, namely, chlorophyllide—




this is an extremely unstable substance which forms green plates.

The enzyme is sensitive to high temperatures, and when boiled with alcohol it is gradually destroyed; its activity is greater at 25° than at 30°.

Chlorophyll appears to be always accompanied by the enzyme, the amount increasing with the amount of chlorophyll, and hence young leaves appear to contain less enzyme than the older ones. In *Pyrus Aucuparia*, *Mellitis Melissophyllum*, *Stachys silvatica*, *Lamium maculatum*, and *Heracleum* the amount of enzyme is comparatively large. *Urtica*, *Avena*, ordinary grasses, *Sambucus*, *Platanus*, *Aspidium*, *Equisetum*, and *Taxus* may be conveniently employed for demonstrating the effect of the enzyme by leaving the tissues in contact with an alcoholic extract of amorphous chlorophyll; practically all the phytol is thereby removed.

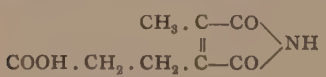
The enzyme is also able to effect the synthesis of phytyl chlorophyllide (amorphous chlorophyll) from chlorophyllide and phytol.

The constitution of this alcohol phytol has been studied by Willstätter and his pupils,* who find it to be an unsaturated compound with a highly branched chain and a double bond between the third and fourth carbon atoms of the chain.

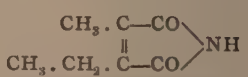
* Willstätter, Schuppli, and Mayer: "Annalen," 1919, 418, 121.

## RELATIONSHIP BETWEEN CHLOROPHYLL AND HÆMOGLOBIN.

With a view to the further elucidation of the constitution of the chlorophyll molecule, especially in regard to the complex to which the carboxyl groups are attached, the oxidation of the porphyrins by means of chromic acid in the presence of sulphuric acid has been studied by Marchlewski* and by Willstätter and Asahina.† These investigations point to the existence of the grouping in the molecule, since the two chief oxidation products are found to be pyrrole derivatives of the formulæ—

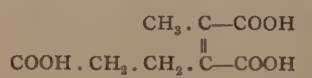


Hæmatinic acid imide



Methylethylmaleinimide

The former substance, which is the imide of a tricarboxylic acid known as hæmatinic acid, of the formula—



has also been obtained from hæmoglobin, the red colouring matter of the blood, and a connexion between hæmoglobin and chlorophyll is thereby established.

The relationship between this hæmatinic acid imide and hæmoglobin is as follows:—

Hæmoglobin is readily hydrolysed by dilute acids or alkalis with the formation of hæmatin; this latter substance contains iron, which can, however, be readily removed by treatment with hydrogen bromide in acetic acid solution,‡ giving an iron free compound hæmatoporphyrin,§ both hæmatin‖ and hæmatoporphyrin on oxidation yield the hæmatinic acid imide mentioned above.

* Marchlewski: "Chem. Zentralbl.," 1902, **1**, 1017.

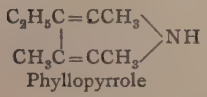
† Willstätter and Asahina: "Annalen," 1910, 373, 227.

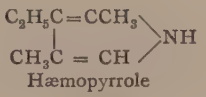
‡ Nencki and Zaleski: "Zeit. physiol. Chem.," 1900, **30**, 423.

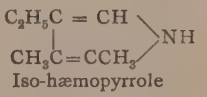
§ It should be noted that chlorophyll derivatives free from magnesium are by analogy called porphyrins: cf. Phylloporphyrin, etc.

‖ Küster: "Zeit. physiol. Chem.," 1899, **28**, 1; 1900, **29**, 185.

Another link between chlorophyll and hæmoglobin is supplied by the fact that Willstätter and Asahina * have obtained from chlorophyll by reduction three pyrrole derivatives :—

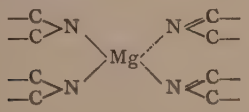

Phyllopyrrole


Hæmopyrrole


Iso-hæmopyrrole

one of which, hæmopyrrole, has also been obtained by the reduction of hæmatoporphyrin.

With regard to the manner in which the magnesium or iron are respectively united to the complex molecules of chlorophyll and hæmoglobin, the following skeletons, involving the assumption of subsidiary valencies, according to Werner and others, have been suggested †:—


Chlorophyll

Hæmatin

In this connexion compare the formula assigned to Aetiophyllin (page 230).

## EXTRACTION OF CHLOROPHYLL.

The usual method of extracting chlorophyll from green tissues consists in first steeping the fresh material in hot water to destroy oxidizing enzymes and then extracting the colouring matter by means of warm alcohol. Willstätter, however, recommends the use of dried in place of fresh material, and extracting by shaking with organic solvents (ethyl or methyl alcohol, ether or acetone) *in the cold.*

The chief advantage in using dried material lies in the fact of its relatively small bulk, 100 grams of stinging nettle leaves, for example, weighing only 25 grams after drying. It has been shown, moreover, that the operation of drying produces no change of any importance in the chlorophyll, since

* Willstätter and Asahina : "Annalen," 1911, 385, 188.
† Willstätter and Fritzsche : *id.*, 1909, 371, 33.

the results obtained from dried material have been repeated and confirmed on fresh material.

On the other hand, organic solvents containing an appreciable amount of water are preferable to the dry solvents. This is attributed by Willstätter to the fact that aqueous solvents dissolve out salts, such as potassium nitrate, from the cell sap, and these affect the state* of the colloidal solution of chlorophyll in the chloroplast, thereby rendering the chlorophyll more easily accessible to the solvent. Moreover, the number of substances going into solution is thereby increased, and the solution is no longer effected by the solvent alone but by the solvent together with the accessory substances.

If dry solvents are used, the extract is much less pure since it contains a larger proportion of carotinoids, lecithins, etc., whose solubilities are very similar to those of chlorophyll.

The following methods of extracting dried or fresh leaves respectively are described by Willstätter :—

1. Half a kilo of dried material is spread on a porcelain Buchner funnel in a layer of not more than 4 to 5 cms. thick, and 1·5 litres of solvent are drawn through this layer by means of a filter pump in the course of half an hour. This filtrate, measuring about 0·9 litre, contains from 4·25 to 4·5 grams of chlorophyll.

The solvent employed may be either 90 per cent (aqueous) alcohol or 80 per cent (aqueous) acetone. The former filters rather more rapidly, but acetone has the advantage over alcohol in preventing the chlorophyll from undergoing what is known as allomerization, a peculiar change which interferes with its power of crystallization, and prevents it giving the phase test.

2. Two and a half kilos of fresh leaves are ground up in a mill and shaken in a bottle with 1·5 litres of acetone to remove water and mucilage and to stop enzyme action. The acetone is then filtered off on a pump; it contains no chlorophyll. The residue is then freed from acetone by filtering on a pump under a pressure of 200 atmospheres, and the resulting hard mass, weighing 0·8 kg., is broken up and ground again. On adding 1·5 litres of acetone the latter becomes diluted to 80 per cent by the water still remaining in the residue; the

* See section on Colloids, p. 283.

mixture is shaken for 5 minutes and a further quantity of 1 litre of 80 per cent acetone is now added. The liquid is filtered off on a pump and the residue treated three times with half a litre of 80 per cent acetone. The total filtrate should measure 3·7 litres and contain 4·7 grams chlorophyll.

In order to ascertain what proportion of the total chlorophyll present has been removed in any particular extraction, another quantity of dried material, say from 100 to 200 grams, may be subjected to an exhaustive percolation with an excess of alcohol until the alcohol comes through colourless. Both extracts are then diluted until 1 kg. of dry powder corresponds to 200 litres of extract and their strengths are compared by means of a colorimeter.

Similarly, a fairly accurate estimate of the amount of chlorophyll present in a solution can be made by colorimetric comparison with a solution containing ·025 gram of pure crystallized chlorophyll dissolved in 1 litre of alcohol. For this purpose the yellow colouring matters must, however, be removed; this is done by allowing the solution to stand for some time with alcoholic potash; the solution is then decanted from the brown resinous deposit which settles on the sides of the vessel, and, after washing the latter with a little more alcohol, the combined alcoholic solutions are diluted with water and extracted with ether to remove the yellow colouring matters.

After suitably diluting with alcohol, the solution is then compared in a colorimeter with the standard chlorophyll solution.

In this way it was found that 1 kg. of fresh stinging nettle leaves containing 25·6 per cent of total solid contained an amount of chlorophyll equivalent to 1·6 grams of the crystalline substance, corresponding, therefore, to 1·6 × 1·38 = 2·2 grams of amorphous chlorophyll.*

The following simple experiments are selected from a number described by Willstätter and Stoll† to illustrate the properties of chlorophyll and the carotinoids:—

1. Grind up 10 grams of fresh stinging nettle leaves with

* The factor 1·38 for converting crystalline into amorphous chlorophyll represents the ratio between the molecular weights of these two substances.

† Willstätter and Stoll: "Untersuchungen über Chlorophyll," Berlin, 1913.

silver sand in a mortar. Cover with 20 c.c. acetone and filter over a pump; wash the residue with more acetone and filter again; the filtrate will contain 0·02 gram chlorophyll.

2. Dried powdered leaves do not part with their colour on treatment with benzene or light petroleum, and only yield chlorophyll very slowly to anhydrous alcohol, acetone, or ether, but may be readily extracted by means of 90 per cent alcohol or 80 per cent acetone yielding a green solution with a strong red fluorescence.

3. Prepare an ethereal solution of chlorophyll as follows: About 15 c.c. of an 80 per cent acetone extract of dried leaves are poured into 30 c.c. of ether contained in a tap funnel and mixed with 50 c.c. water. The ethereal solution rises to the surface. It should be washed four times with 50 c.c. of water each time by carefully allowing the water to run down the side of the funnel without shaking. If a 30 per cent methyl alcoholic solution of potash is now run under the ether layer a brown colour is produced at the junction of the two liquids. The colour gradually changes to olive-green and finally back to the original green. The reaction, which is known as the "Phase Test," is due to the saponification of the chlorophyll with formation of the potassium salt of chlorophyllin. Consequently on dilution with water the green colour remains in the aqueous layer and is no longer soluble in ether.

4. Shake vigorously 5 c.c. of an ethereal solution of chlorophyll (prepared as above) with 2 c.c. of concentrated methyl alcoholic potash. When the green colour has returned dilute with 10 c.c. water, added in portions, and add a little more ether. On shaking, two layers separate; the lower aqueous alkaline layer contains the chlorophyll while the ether contains carotinoids.

5. To separate xanthophyll from carotin wash the ethereal solution of these two substances obtained from previous experiment with a little water and evaporate to 1 c.c. Dilute with 10 c.c. of light petroleum, and shake up two or three times with 10 c.c. of 90 per cent methyl alcohol until the latter is no longer coloured. The methyl alcohol will contain the xanthophyll while the carotin will be in the light petroleum.

## THE CAROTINOIDS OR YELLOW PIGMENTS ACCOMPANYING CHLOROPHYLL.

In addition to chlorophyll four pigments which are insoluble in the cell sap occur in plants either in a relatively pure form in chromoplasts, or associated with chlorophyll in the chloroplasts; they are carotin, lycopin, xanthophyll, and fucoxanthin. Of the carotinoids, the most important are carotin and xanthophyll, which were at one time supposed to be identical. From the researches of Arnaud* and Willstätter and Mieg† there is no doubt that xanthophyll and carotin are different substances. Willstätter and Escher,‡ moreover, have isolated from the fruits of the tomato a yellow pigment, lycopin, isomeric with carotin. It differs, however, from carotin in some of its physical properties and in the amount of oxygen it takes up on oxidation. While according to some authors§ the carotinoids have their origin in a particular kind of elongated mitochondrium, they are, according to other workers, to be regarded as decomposition products of chlorophyll.

The colour changes in fruits and leaves are, according to Lubimenko,‖ due to two classes of compounds which he terms lycopinoids and rhodoxanthinoids, each of which may be further subdivided according to their chemical and physical properties. The lycopinoids may be either amorphous or crystalline, and several may be associated in one chromoleucite; during fruit ripening the amorphous lycopinoids gradually change into the crystalline lycopin. The lycopinoids are more widely distributed than are the rhodoxanthoids; temperature is an important factor in their formation and oxygen is an essential.

* Arnaud: "Bull. Soc. Chem.," 1887, **48**, 64.

† Willstätter and Mieg: "Annalen," 1907, **355**, 1.

‡ Willstätter and Escher: "Zeit. physiol. Chem.," 1910, **64**, 47.

§ Guilliermond: "Compt. rend.," 1917, **164**, 232, 407, 609, 643; Mottier: "Ann. Bot.," 1918, **32**, 191.

‖ Lubimenko: "Compt. rend.," 1914, **158**, 510.

### CAROTIN $C_{40}H_{56}$.

This pigment is widely distributed and, as has already been mentioned, is generally associated with chlorophyll in the chloroplasts. It also occurs in various forms, amorphous or crystalline, in various parts of many plants. The colour of yellow or orange petals is not infrequently due to it, e.g. the corona of the common Narcissus, *N. Poeticus;* similarly the presence of innumerable small intracellular crystals of carotin are responsible for most of the colour of the root of the carrot, and so also is the tint of many fruits where the carotin is often in amorphous granules.

With regard to the physiological significance of carotin, the work of Tammes and Kohl* shows that carotin absorbs certain rays of radiant energy which can be made use of in photosynthesis.

In addition to this there is the possibility that carotin may be of importance in respiration, acting in a manner comparable to the hæmoglobin of the blood.†

The possible function of the carotinoids in assimilation has already been referred to on page 228.

In those cases where a large amount of carotin occurs in organs of storage, such as the roots of the carrot, it may be of value as a reserve food-material. Finally, where the colours of flowers are due to its presence, carotin is important in the floral biology.

Carotin is insoluble in water and very slightly soluble in acetone or cold alcohol; in hot alcohol it is more soluble; and in ether, chloroform, light petroleum, and carbon bisulphide it is readily soluble. The colour of the solution varies from yellow to red; on crystallization flat reddish-yellow plates are formed which exhibit the phenomenon of dichroism, being orange-red by transmitted light and greenish-blue in reflected light.

According to Willstätter,‡ carotin may be extracted from stinging nettle leaves by light petroleum; it has the molecular formula $C_{40}H_{56}$, and is probably identical with the sub-

* Kohl: "Ber. deut. bot. Gesells.," 1906, **24**, 222.
† Arnaud: "Compt. rend.," 1889, **109**, 911.
‡ Willstätter and Mieg: "Annalen," 1907, **355**, 1.

stances erythrophyll and chrysophyll described by Bougarel and Schunck respectively.

It absorbs 34·3 per cent of its weight of oxygen, being converted into a colourless substance. With iodine it forms the compound $C_{40}H_{56}I_2$, which crystallizes in dark violet prisms.

### XANTHOPHYLL $C_{40}H_{56}O_2$.

This substance is closely related to carotin, having the molecular formula $C_{40}H_{56}O_2$. Ewart * has, indeed, shown that xanthophyll may be converted into carotin by the action of zinc dust or magnesium powder and water.

It is a neutral substance, reacting neither as an alcohol nor as an acid.

It absorbs 36·55 per cent of its weight of oxygen, and forms an additive compound with iodine of the formula $C_{40}H_{56}O_2I_2$, which crystallizes in dark violet tufts.

The more important physical constants and solubilities of carotin and xanthophyll are given in the appended table, compiled by Willstätter :—

| | *Carotin.* | *Xanthophyll.* |
|---|---|---|
| Appearance . . . | Copper coloured leaflets. | Pleochroic dark reddish-brown plates. |
| Colour by transmitted light . . . . | Red. | Yellow to orange. |
| Melting-point . . . | 167·5-168°. | 172°. |
| Solubility in light petroleum . . . | Appreciably soluble. | Insoluble. |
| Solubility in alcohol . | Practically insoluble in cold; very sparingly soluble in hot. | Sparingly soluble in cold; fairly readily soluble in hot. |
| Solubility in acetone . | Very sparingly soluble. | Readily soluble. |
| Solubility in carbon disulphide . . . | Very readily soluble. | Sparingly soluble. |

### FUCOXANTHIN $C_{40}H_{54}O_6$.

This substance was first isolated from fresh brown algæ by Willstätter and Page.† It is more difficult to extract this substance from dried algæ. Fucoxanthin is a brownish-red substance, which crystallizes from methyl alcohol or light petroleum, and melts at 159·5 to 160·5°. It absorbs iodine

* Ewart: "Proc. Roy. Soc.," 1915 [B], 89, 1.
† Willstätter and Page: "Annalen," 1914, 404, 237.

to form a compound $C_{40}H_{54}O_6I_4$. Unlike carotin and xanthophyll, which are neutral substances, fucoxanthin has basic properties, and forms blue salts with hydrochloric and sulphuric acids.

FURTHER LITERATURE.

Perkin and Everest: "The Natural Organic Colouring Matters," London, 1918.
Schryver: "Science Progress," 1909, 3, 425.
Stahl: "Zur Biologie des Chlorophylls," Jena, 1909.
Willstätter and Stoll: "Unters ü. Chlorophyll," Berlin, 1913.

## ANTHOXANTHINS.

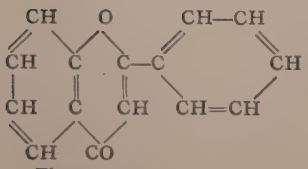
### FLAVONES AND XANTHONES.

Under the headings of Flavones and Xanthones (two words derived from the Latin and Greek for yellow) are included a number of yellow pigments occurring in the vegetative organs and in the petals of many plants. Owing to their close relationship to the blue colouring matters known as Anthocyanins, Willstätter and Everest,* have proposed the adoption for them of the generic term, Anthoxanthin, at first suggested by Marquart in 1835. These yellow pigments are often of considerable economic value as dye-stuffs. They occur naturally in combination with rhamnose or glucose as glucosides and in some cases uncombined, and frequently are also associated with tannins.

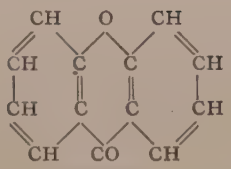
The anthoxanthins are widely distributed amongst the higher plants; they are most abundant in plants which grow under conditions of high insolation, unless there be a protection in the form of hairs or thick cuticle. For this reason they are looked upon as affording a protection against the light rays of shorter length. There is sometimes an interchange between the anthoxanthins and anthocyanins, thus young plants often contain red anthocyanin, which gives place to a colourless flavone in the mature stage; at leaf-fall the anthocyanin may reappear.†

The mother substances from which all these compounds are derived and from which they derive their name are the two compounds Flavone and Xanthone, both of which contain the pyrone nucleus (see p. 246).

* Willstätter and Everest: "Annalen," 1913, 401, 189.
† Shibata and Nagai: "Bot. Mag. Tokio," 1916, 30, 149.



Flavone

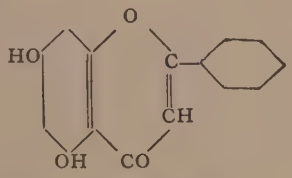


Xanthone

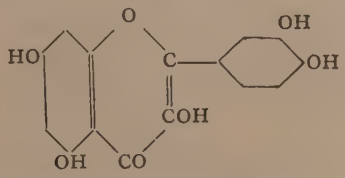
YELLOW COLOURING MATTERS DERIVED FROM FLAVONE.

There are quite a considerable number of yellow substances occurring in plants derived from flavone, but only a few representative ones will be mentioned here in order to give some idea of the constitution of these compounds.

*Chrysin*, or dihydroxy-flavone, is a yellow colouring matter occurring in several varieties of poplar, such as *Populus nigra* and *P. pyramidalis*.

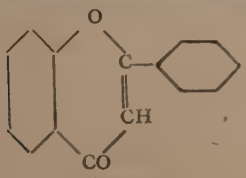



*Quercetin*, or tetrahydroxy-flavonol *

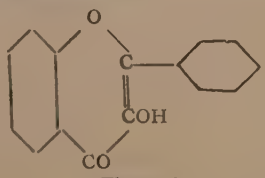



occurs with rhamnose in the form of a glucoside in the bark of *Quercus tinctorius*, in the leaves of the horse-chestnut and hop, and in many other plants. Quercetin, in the uncombined

* Flavonol is the hydroxyl derivative of flavone; the relationship between the two substances is shown by the following formulæ :—



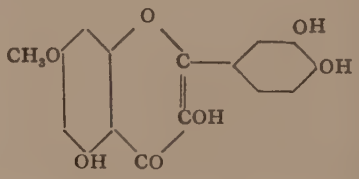
Flavone



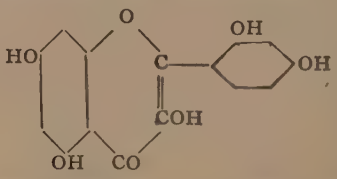
Flavonol

state, also is found in the bark of *Pyrus Malus* and in the leaves of *Thea sinensis*, *Arctostaphylos Uva-ursi*, *Acacia catechu*, and many other plants.

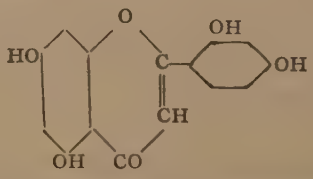
*Rhamnetin*, the monomethyl ether of tetrahydroxy-flavonol or quercetin monomethyl ether, occurs in the dried berries of *Rhamnus cathartica* and *R. tinctoria*, both of which are used for dyeing cotton.




*Morin.*—This substance, which is isomeric with quercetin, occurs in the wood of *Morus tinctoria* (yellow wood), where it is accompanied by another colouring matter, maclurin, sometimes called moringatannic acid (see p. 202).




*Luteolin.*—This is the yellow colouring matter of *Reseda luteola*, known as "weld"; it is also contained in *Genista tinctoria* or Dyer's broom.

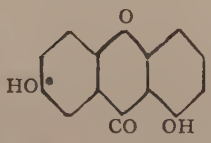



Other members of this group of substances are *Apigenin*, occurring in *Apium petroselinum*, and *Fisetin*, occurring in *Quebracho colorada*, and *Rhus cotinus* or Dyer's sumach.

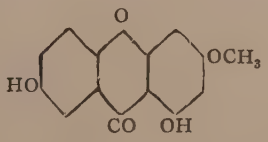
### YELLOW COLOURING MATTERS DERIVED FROM XANTHONE.

There are as yet only three colouring matters known to belong to this group, one of which, euxanthone, does not occur

in plants, but in Indian yellow obtained from camel's urine; it has the formula




*Gentisin* is a yellow colouring matter occurring in *Gentiana lutea.*




*Datiscetin* occurring in the form of a glucoside, *Datiscin*, in *Datisca cannabina.*

### *Properties of Anthoxanthins.*

1. These colouring matters are mostly yellow crystalline solids.

2. In water the crystals are hardly soluble, in acids they dissolve readily giving yellow to red solutions, and in alkalies they also are soluble, yielding the same coloured solutions.

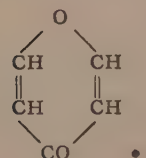
3. From their solutions they may be precipitated by lead acetate, the precipitate being yellow, orange, or red.

4. Aniline or toluidine nitrate and potassium nitrite give a cinnabar red precipitate.

5. With ferric chloride a dull green or sometimes a red-brown coloration results.

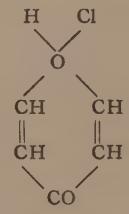
6. On fusion with alkali, decomposition ensues, phloroglucinol and protocatechuic acid being commonly formed, and sometimes resorcinol, resorcylic, or hydroxybenzoic acids.

The solubility of the anthoxanthins in acids is due to the peculiar basic properties of the oxygen atom taking part in the ring formation. The basic nature of the oxygen atom in such circumstances was first observed in the case of the simpler substance pyrone



Pyrone

which dissolves in hydrochloric acid, forming an additive compound of the formula



the oxygen becoming tetravalent. Such additive compounds of anthoxanthins with acids are easily dissociated and do not occur in plants, though it will be seen on page 248 that in the case of the anthocyanins analogous compounds do actually occur naturally.

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Kostanecki: "Bull. soc. chim. Paris," 1903, [3], **29**, i-xxxvii.

Perkin, A. G., and others: "J. Chem. Soc. Lond.," 1895, **67**; 1896, **69**; 1897, **71**; 1898, **73**; 1899, **75**, etc.

Wheldale: "Proc. Camb. Phil. Soc.," 1909, **15**, 137; "Biochem. J.," 1914, **8**, 204, etc.

## ANTHOCYANINS.

Occurring in the cell sap, often in sufficient quantity to mask entirely the green colour of the chlorophyll, are a number of pigments, other than chlorophylls, belonging to various classes of chemical compounds.

Under the collective heading of Anthocyanin are included a number of such pigments of a blue, red, or violet tint occurring in the flowers, fruits, or leaves of many plants.* The first representative of the class to be isolated in a state of purity by Willstätter and Everest† was Cyanin, the blue

* An historical account of our knowledge of these pigments is given by Everest in "Science Progress," 1915, 9, 597. See also Wheldale: "The Anthocyanin Pigments of Plants," Cambridge, 1916.

† Willstätter and Everest: "Annalen," 1913, **401**, 189.

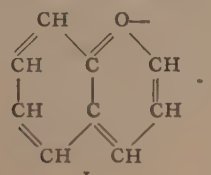
colouring matter of the cornflower *Centaurea cyanus* and of *Rosa gallica ;* closely related to this substance are the anthocyanins of the cranberry (idæin), the bilberry (myrtillin), of blue grapes (œnin), of *Delphinium consolida* (delphinin), and of *Pelargonium zonale*, var. *meteor* (pelargonin), *Althaea rosea* (althaein), *Malva sylvestris* (malvin).

The anthocyanins are all glucosides, and on hydrolysis yield one or more molecules of a carbohydrate, together with a so-called anthocyanidin, which compound, unlike the parent substance, is soluble in amyl alcohol. On these facts is based what is known as the *anthocyanin reaction*, according to which a solution of the substance in sulphuric acid yields nothing to amyl alcohol, but after hydrolysis the resulting anthocyanidin may be extracted from the solution quantitatively by amyl alcohol.

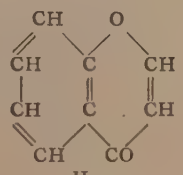
The products obtained by the hydrolysis of the various anthocyanins so far investigated are given in the following list :—

| | |
|---|---|
| Cyanin yields Cyanidin | + 2 mols. glucose. |
| Idæin yields Cyanidin | + 1 mol. galactose. |
| Œnin yields Œnidin | + 1 mol. glucose. |
| Myrtillin yields Myrtillidin | + 1 mol. glucose. |
| Delphinin yields Delphinidin | + 2 mols. glucose + 2 mols. p. hydroxybenzoic acid. |
| Pelargonin yields Pelargonidin | + 2 mols. glucose. |

The anthocyanidins or non-carbohydrate moiety of the anthocyanins are derivatives of benzo-pyrilium which, as may be seen from the appended formula I.,



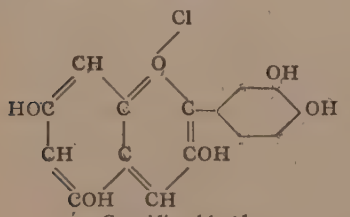

I.



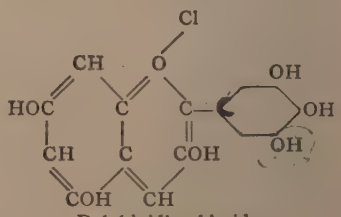

II.

is closely related to benzo-pyrone II., the mother substance of the flavones. Both these substances contain a so-called basic oxygen atom which by becoming tetravalent can form additive compounds with acids producing oxonium salts. These salts in the case of the flavones are not stable and do not occur in the plant, but the anthocyanidins yield stable oxonium

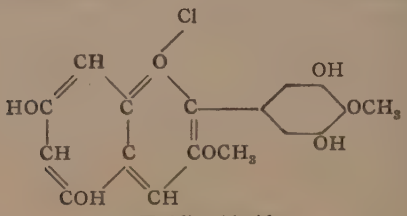
salts of the type illustrated by the following structural formulæ of a few anthocyanidins:—




Cyanidin chloride

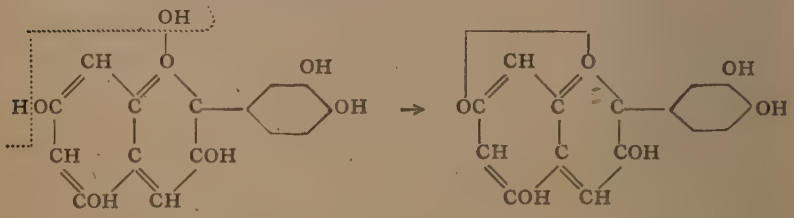



Delphinidin chloride




Œnidin chloride

It will be seen from these formulæ that the oxygen is tetravalent and that the molecules contain phenolic hydroxyl groups capable of forming salts with alkalies. Moreover, by replacing the chlorine by the hydroxyl group on addition of caustic alkalies the possibility of eliminating water from the molecule arises as follows:—




These considerations explain the colour variations produced by the same cyanidin occurring in the same or in different flowers, it having been found, for example, that the same cyanidin was responsible for the colour of the cornflower and of the rose.*

Thus when combined, as in the case of cyanidin chloride, with mineral acid or in the plant with organic acids, the com-

* Willstätter and Nolan: "Annalen," 1915, 408, 1.

pound has a red tint. When treated with alkali, blue metallic salts are formed, while the arrangement shown in the formula II. represents a neutral compound having a violet tint. The neutral violet tinted delphinin has been isolated from *Delphinium consolida* by Willstätter and Mieg,* and has been shown to turn blue with alkali, and red with acids; the colour would therefore appear to act as an indicator in the plant itself, showing whether the cell sap is neutral acid or alkaline.

The colours of different flowers are not necessarily due to the same anthocyanin. Willstätter found three pigments in the cornflower: purple, an acid flavone derivative; blue, the potassium salt of the purple; and red, an oxonium type of salt of anthocyanin with an acid present in the cell sap. Wheldale and Bassett,† on the other hand, conclude that the anthocyanins of *Antirrhinum* must be different from those of *Centaurea*, since in the latter plant no oxonium salts are produced with mineral acids; further, the dissimilarity in the range of colour varieties thrown by the plants in question also suggest the same conclusion. If, in *Antirrhinum*, the anthocyanins are derived from flavones, their origin must in part be due to oxidation since the red and magenta anthocyanins contain a much higher percentage of oxygen than do flavones. The anthocyanin molecule is apparently larger than the flavone, so that if the chromogen is flavone, its condensation must be either the combination of two flavone molecules or one flavone molecule with one or more molecules of phenol, or an aromatic acid, etc.

Sometimes it is observed that the leaves of certain plants when first they unfold are bright red and that in a few days this colour fades away and the green colour is seen. Noack‡ has investigated this phenomenon in *Polygonum compactum*, and thus explains it: by the action of an enzyme the anthocyanin is converted into anthocyanidin and a sugar. The anthocyanidin is then converted into a colourless pseudobase which may be oxidized to a yellow pigment. In the process,

* Willstätter and Mieg: "Annalen," 1915, 408, 61.
† Wheldale and Bassett: "Biochem. Journ.," 1914, 8, 204.
‡ Noack: "Zeitsch. Bot.," 1918, 10, 561.

light is of importance; the pseudobase is due to the photochemical reduction of the oxidation product of the original pigment. In the dark, on the other hand, anthocyanidin is oxidized, a process accelerated by heat.

### CONNECTION BETWEEN ANTHOCYANINS AND ANTHOXANTHINS.

A comparison of the formula of cyanidin chloride on page 248 with that of quercetin on page 243 reveals a close relationship between these two substances, and consequently between the flavones or anthoxanthins and the anthocyanins. Theoretically it should be possible to pass from anthoxanthins to anthocyanins by reduction, or conversely from anthocyanins to anthoxanthins by oxidation. In the plant no doubt this is effected readily enough by enzymes, but in the laboratory it is more difficult, and so far the only transformation effected has been the reduction of quercetin to cyanidin.* These facts provide a confirmation of the views put forward by Wheldale and others previous to this definite experimental evidence. Further evidence for the close relationship between the two classes of compound is provided by the fact that cyanidin is isomeric with luteolin and fisetin while delphinidin is isomeric with quercetin and morin.

### EXTRACTION OF ANTHOCYANINS.

The method of extracting anthocyanins varies with the material employed.† The method recommended in the case of grape skins is as follows: Extract the skins in the cold with glacial acetic acid and precipitate the dark red filtrate with ether; by heating the deposit so obtained with a solution of picric acid a crystalline picrate is formed which separates out on cooling.

* Willstätter and Mallison: "Sitzungsber. K. Akad. Wiss., Berlin," 1914, 769.

† Cf. Willstätter and Mieg: "Annalen," 1915, 408, 61 also Willstätter and Bolton: *id.*, 1915, 408, 42.

### OCCURRENCE.

The occurrence of a red, blue, or purple pigment, either dissolved in cell sap—the exact colour depending on the acid, alkaline, or neutral reaction of the cell sap—or, less frequently, in the form of needle-shaped crystals, as in the case of *Delphinium ajacis*, is a common phenomenon, and is generally ascribed to the presence of the pigment anthocyanin. It is, however, doubtful whether all such colorations are due to anthocyanins; thus Molisch found that the red colour assumed by the leaves of the aloe, on exposure to high insolation, is due to the formation of carotin within the chloroplasts.

The presence of anthocyanin is due to many causes, light, especially when of high intensity, being important. For example, apples and other fruits and also the vegetative organs of certain plants will not assume a red colour if kept in darkness; on the other hand, light does not appear to be of such importance in the case of the roots of the beet.

In other instances the aerial vegetative organs of many varieties of plants, e.g. certain Chenopodiaceæ, are characterized by a red colour the presence of which is seemingly independent, or nearly so, of external conditions. Thus *Salicornia ramosissima* may be found in two forms, one apple green and the other crimson, the intensity of which varies in different years. In such cases there is good reason for supposing that these colours are of an hereditary nature and come true from seed. The same also appears to be true for different forms of beet which are used for horticultural purposes. On the other hand, in the familiar example of the copper beech this is not so, the copper-coloured foliage, due to the combined effect of a red cell sap and the green of the chlorophyll, first originated, it is stated, as a sport and is propagated by means of cuttings.

### *Properties.*

The chief physical property of anthocyanin is its absorption spectrum. Engelmann found that it is complementary to that of chlorophyll, the main absorption bands being in the yellow and yellow-green, with minor ones in the blue end of the spectrum.

Questions relating to the energy relationship between this and other pigments and chlorophyll are outside the scope of the present consideration; it may be mentioned, however, that it has been stated that leaves containing anthocyanin have relatively less chlorophyll than those which have no red pigment.

According to Pick and others, anthocyanin is commonly associated with tannins, for a red sap is characteristic of tannin-containing plants, and the precipitate appearing in the palisade cells of *Hydrocharis* on treatment with caffeine and antipyrine closely resembles the precipitates given by the same reagents with tannin. Plants in which this particular pigment does not occur are free from tannin.

The appearance of anthocyanin is closely related to the sugar-content of the tissues in which it occurs.

Ewart * has pointed out that in the case of *Elodea canadensis* and other aquatic plants the red dye will appear provided the plants be immersed in a weak solution of sugar and exposed to strong sunlight at ordinary temperatures, whilst the red colour does not appear if the plants be grown in water or in diffuse daylight.

These experiments of Ewart were much extended by Overton,† who used *Hydrocharis* and other plants. He found that, in addition to the presence of sugar, light and temperature were important factors. If the temperature be low, but above freezing-point, then the formation of the red pigment will be promoted, which accounts for the red colour prevalent in alpine plants, since under their conditions of existence sugar tends to accumulate rather than starch. This also is true for arctic plants in which, according to the observations of Wulff,‡ the leaves are very frequently sugar leaves, and are commonly characterized by the presence of anthocyanin.

In the case of *Hydrocharis* grown in water culture, Overton found that when the temperature and the intensity of light were so balanced that no colour was formed, the addition of 2 per cent of invert sugar caused its appearance in three days, not only in the young leaves but also in the old ones.

* Ewart: "Journ. Linn. Soc., Lond., Bot.," 1895-7, **31**, 445; "Ann. Bot.," 1897, **11**, 461.

† Overton: "Nature," 1899, 59, 296; "Jahrb. Wiss. Bot.," 1899, 33.

‡ Wulff: "Botanische Beobachtungen aus Spitzbergen," Lund., 1902.

Other aquatic plants behave similarly, but in the case of cut shoots of lilies the red pigment only developed provided sugar were added to the culture solution.

Further experiments showed that the red colour is not formed in those plants, in which the pigment was restricted to the epidermis, when cultivated in sugar solution. Success only resulted in those cases where the colouring matter occurred in the mesophyll.

In view of these facts Overton considered that anthocyanin had some connexion with tannins, and was probably a glucoside (p. 247). A similar view was held by Combes,* who called attention to the facts that, as compared with the green leaves, the red autumnal leaves of *Ampelopsis hederacea*, etc., contain more sugars and glucosides, the amount of anthocyanin varying directly as the sugars and glucosides; that the dextrins diminish as the sugars and glucosides increase; and that the formation of anthocyanin is not apparently dependent on the insoluble carbohydrates. For these and other reasons he concluded that the substance in question was probably a cyclic glucoside which arose, not at the expense of pre-existent sugars and glucosides nor of chromogens, but in the ordinary course of constructive metabolism; also, he concluded, it was only formed provided that oxygen be present.

The observations of Boodle† also indicate the relationship between anthocyanin and sugar. He found that in the leaves of *Rheum*, some of the veins of which had been accidentally severed, anthocyanin made its appearance in the mesophyll supplied by these veins. Boodle then experimented with species of *Oenothera;* all the species examined were not equally responsive, but in the case of *O. biennis* the severance of the midrib at about its middle caused the whole region distal to the cut to become red provided the plant were exposed to daylight. The operation interrupted the path of transport of carbohydrate from the leaf, so that sugar accumulated above the cut, and it is this concentration of soluble carbohydrates which leads to the development of anthocyanin. In this connexion the work of Linsbauer‡ may be referred to.

* Combes: "Ann. Sci. Nat. Bot.," 1909, **9**, 274.
† Boodle: "New Phytologist," 1903, **2**, 207.
‡ Linsbauer: "Oestr. Bot. Zeit.," 1901, **51**, 1.

That the presence of anthocyanin is connected with nutritive processes there can be no doubt, but other substances besides sugar may come into play; thus Dendy observed that the addition of protein to the water, caused green plants of *Hæmatococcus* to turn red.

Finally, the work of Wheldale* on colour inheritance in flowers, points to the conclusion that anthocyanin is a product of the action of an oxidase upon glucosidal flavones, a view which is entirely borne out by the chemical evidence outlined on page 246.

### *Reactions.*

1. Soluble in water, alcohol, and ether.
2. The solution is coloured according to the reaction, red in the presence of acid and blue when the medium is made alkaline.
3. Strong alkalies decolorize the solution.
4. Basic lead acetate gives a green precipitate.
5. With salts of iron, a green or blue coloration results.

### *Physiological Significance.*

In considering the physiological significance of anthocyanin it must be borne in mind that the substance may occur in almost any organ of a plant, from the root to the flower, and in plants very remote phyletically one from the other; and that chemically this pigment may not always be exactly the same. Further, as its appearance seemingly depends upon the immediate metabolic condition of the plant, and so in some cases may be sporadic, whilst in other instances it is characteristic of the species or variety or form, care must be exercised in ascribing to it a definite function. Its presence may be due to nothing more than the particular metabolic sequence; in other words, an accident, which, in some examples may be a lucky one for the plant.

It is, of course, not surprising to find that several opinions have been put forward to explain its presence.

According to Pick the dye is a filter to separate from the light entering the leaf certain rays which would be deleterious

* Wheldale: "Proc. Camb. Phil. Soc.," 1909, 15, 137; "Journ. Genet.," 1911, 1, 10.

to the translocation of the starch. Keeble found that in leaves which had the dye on one side but not on the other, the difference in temperature due to the anthocyanin was about 2° C., and he concluded that it may be of value as a protective mechanism against the heating effect of strong sunlight.

Stahl* thought that it absorbs heat and so increases transpiration, especially in the case of tropical plants. Ewart points out that, although this might sometimes be of value, if it were the primary function it would naturally be expected that anthocyanin would absorb the heat rays more particularly. Also Ewart cites his observations on *Elodea* against Stahl's view, and remarks that "since the plants [*Elodea*] are submerged, it cannot possibly be for the purpose of increasing what is non-existent, i.e. transpiration, nor can it perceptibly raise the temperature of a submerged plant". The first argument may no longer be valid, for it appears that a transpiration current may exist in submerged aquatic plants.†

Ewart believes that anthocyanin is to protect the chlorophyll against the action of too strong light. He gives experimental data in support of his view, and cites the observations of Schröder and Klebs to the effect that the pigment is of importance in protecting the chlorophyll in *Hæmatococcus* and the resting spores of many Algæ.

Ewart does not think that the pigment is an accidental occurrence in all cases, for in *Elodea* it is not formed in diffuse light; on the other hand, in the beetroot it probably has no special function, and may be a waste product of metabolism.

Shibata‡ and his colleagues found that derivatives of flavones are almost universally distributed in sub-aerial plants, alpine and tropical plants particularly so. They conclude that the presence of these substances, especially when in the peripheral tissues, absorb the ultra-violet rays, and thus are protective. Rosenheim§ supports this view since he found that *Leontopodium alpinum*, the Edelweiss, when grown in London, contained but a quarter the amount of the substances in question as compared with plants grown in the Alps.

* Stahl: "Ann. Jard. Bot. Buitenzorg," 1896, **13**, 137.

† See Thoday and Sykes: "Ann. Bot.," 1909, **23**, 635.

‡ Shibata: "Bot. Mag. Tokio," 1915, **29**, 118; Shibata, Nagai, and Kishida: "J. Biol. Chem.," 1916, **28**, 193.

§ Rosenheim: "Biochem. Journ.," 1918, **12**, 283.

According to Buscalioni and Polacci* anthocyanins may increase the osmotic forces of the cell, but they are careful to point out that they may perform many functions in different plants.

Wulff considers that the pigment is of value in the absorption of extra radiant energy, and is of great importance in arctic plants, for instance, which live under conditions unfavourable for metabolic activities.

Combes holds views similar to those of Palladin, that anthocyanin is closely connected with respiration. If the sugar content increases the rate of respiration is accelerated, and this leads to the formation of the pigment.

It may be remarked that most of the above opinions were put forward before the work of Palladin on respiration and the relationships between pigments and enzymic activity appeared. And, in view of this, some of the earlier experiments appear to require reconsideration from Palladin's point of view.

## PHYCOERYTHRIN.

Phycoerythrin is a red pigment commonly occurring in red sea-weeds, especially when growing in deep water. It has been investigated by Hanson,† on whose account the following description is based:—

Phycoerythrin is easily soluble in water, giving a rose-coloured solution which exhibits a well-marked orange fluorescence; the spectrum shows that the chief absorption is that of the blue-green rays.

### *Preparation.*

To prepare a solution of phycoerythrin the red sea-weed, *Ceramium rubrum*, which is one of the best to use, is washed in ordinary water to free it from sea salts and adhering sand. It is then soaked in distilled water; in two days most of the pigment will have diffused out. The solution is filtered through glass wool and a few drops of eucalyptus oil added as an antiseptic, for putrefaction soon sets in.

* Buscalioni and Polacci: "Atti. Inst. Bot., Pavia," 1904, II., 8, 1, 135.
† Hanson: "New Phytologist," 1909, 8, 337.

It is a matter of great difficulty to obtain a pure sample of phycoerythrin, for, in an aqueous solution, it passes over into an irreversible gel,* even when kept at 0° C. This, of course, renders ordinary filtration extraordinarily slow, and thus increases the difficulty of purification.

The solid phycoerythrin may be prepared from the aqueous solution by concentrating it under reduced pressure at a temperature not higher than 38° C.; any precipitate which comes down during this process must be filtered off. Methylated spirit is then added to the concentrated solution until the fluorescence disappears. The precipitated phycoerythrin is allowed to settle and the more or less clear supernatant fluid is filtered off, again treated with alcohol, and filtered. The operation is repeated until the red colour has entirely disappeared from the solution. The precipitates are washed by decantation with 70 per cent alcohol; the pigment, in a pasty mass, is placed in a clock glass and dried in a vacuum.

*Reactions.*

The following reactions are among those recorded by Hanson:—

1. Phycoerythrin is precipitated from its solution by alcohol, by small quantities of mercuric chloride, and by saturation with ammonium sulphate and magnesium sulphate.

2. When dilute acids are added gradually, the fluorescence first disappears, leaving a somewhat opalescent solution of a lilac-pink tint. After the lapse of two days a pink precipitate comes down.

3. Ammonium hydrate in small quantities removes the fluorescence; in excess, a yellowish-brown coloration results.

4. Caustic soda or potash in small quantities causes the red colour to disappear, the solution turning opalescent and yellowish-brown in colour; on standing, a brownish precipitate comes down.

5. The solution is immediately decolorized by bleaching powder, bromine water or a solution of iodine in potassium iodide.

6. Mercuric chloride solution in small quantities gives a lilac-grey precipitate, the solution then being yellowish in colour.

* See Section VIII., on the Colloidal State.

7. Ferric chloride gives a pinkish-brown precipitate.

8. Boiled with nitric acid a yellow colour results which turns to orange on adding an excess of ammonia.

9. Boiled with Millon's reagent a deep red colour results.

10. The addition of a caustic soda solution followed by a drop or two of dilute copper sulphate gives a greenish tint.

11. Digestion with pepsin, in the presence of hydrochloric acid, has no result.

12. On digestion with trypsin in the presence of sodium carbonate, the phycoerythrin loses its colour, and the solution contains a very small amount of leucin, but no tyrosin.

13. On hydrolysis with acids, tyrosin is found in very small amounts, but leucin occurs in greater quantities.

From these and other facts it is concluded that phycoerythrin is a colloidal nitrogenous substance allied to the proteins; it is not a true protein, since its nitrogen content is too low and it does not give the biuret reaction. It is impossible to say anything more definite regarding its chemical nature until it has been prepared in a pure state in quantities sufficient for analysis.

Physiologically, phycoerythrin acts as a pigment complementary to chlorophyll. It absorbs the blue-green rays, and degrades them to yellow and red light of just those wavelengths which the chlorophyll can absorb.

## PHYCOPHAEIN.

As is well known, a brown colouring matter may be extracted by water from the Phæophyceæ and other brown Algæ. Hitherto this has generally been considered to be due to the presence within the cells of a definite colouring matter of a protein nature. According, however, to the work of Molisch * and Tswett,† this is not the case. The brown colouring matter is really due to post-mortem changes, the oxidation of a water soluble chromogen. An extract prepared with distilled water is at first colourless, but will turn yellow if the solution is made alkaline in reaction, e.g. by tap water, and finally brown owing to oxidation. If the reaction be made acid decolorization will result. With regard to the chemistry of this substance little, if anything, is known.

* Molisch: "Bot. Ztg.," 1894, **52**, 181; 1895, **53**, 131; 1905, **63**, 131.
† Tswett: "Ber. deut. bot. Gesells.," 1906, **24**, 235.

With regard to the physiological significance of these pigments in the Algæ, the work of Gaidukov * on complementary chromatic adaptation may be consulted.

## RESPIRATION.

Although it is not proposed to enter into a detailed consideration of the phenomena of respiration here, brief mention may be made of Palladin's † conceptions on the subject on account of the rôle he ascribes to colouring matters and allied substances in respiratory activity.

Occurring in plants are pro-chromogens which may be glucosides or may be decomposition products of proteins. These pro-chromogens, by the action of enzymes, give origin to chromogens.

Chromogens are widely distributed in the vegetable kingdom, in fact are universally present in those parts of plants which are respiring; they, however, vary in amount at different seasons of the year and according to the physiological condition of the plant. For instance, in the spring they occur in abundance in the young leaves, and in the autumn the old and dead leaves also contain much owing to the lack of co-ordination of enzymic activity.

At other times the amount of chromogens is not very great, but may be increased by suitable treatment. Thus Palladin found that leaves kept for a week in a strong solution, 20 to 30 per cent, of cane sugar showed a great increase, whereas leaves kept in distilled water and also untreated leaves of the plant showed no such increase. A bright illumination also increases the amount of chromogens.

The chromogens are acted upon by oxidases in the presence of oxygen and yield pigments which may be reduced by reducing enzymes or reductases. Carotin and Xanthophyll provide convenient examples. Carotin, $C_{40}H_{56}$, is acted upon by an oxidase and converted into Xanthophyll, $C_{40}H_{56}O_2$, which in turn is acted upon by a reductase yielding carotin. This action is comparable to that of the hæmoglobin in the blood, and in fact Palladin has termed all such respiratory pigments

* See Blackman: "New Phytologist," 1904, **3**, 237.

† Palladin: "Ber. deut. bot. Gesells.," 1908, **26a**, 125, 378, 389; 1909, **27**, 110.

of plants, no matter what their composition may be, phyto-hæmatins.

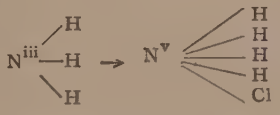
It does not follow that all definite coloured compounds are formed during respiration; it all depends on the relative activities of the oxidases and the reductases. A pigment will make its appearance provided the oxidases are the more potent, but if the reductases are the more active no pigment will appear.

The method of indicating the presence of a chromogen is obvious; the material to be examined is extracted and heated to a degree of temperature sufficient to destroy any enzymes present. To this preparation is added peroxidase and hydrogen peroxide; if a chromogen were present originally, then a coloration will result, usually brown, red, or purple.

# SECTION VII.

## NITROGEN BASES.

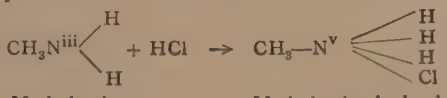
AMMONIA is said to have basic properties because it can form salts by combining with acids. This salt formation, which may be illustrated by the conversion of ammonia into ammonium chloride, is due to the unsaturated nature of the trivalent nitrogen atom, and its tendency to assume the pentavalent condition.



Ammonia  Ammonium chloride

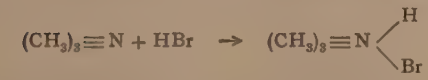
The replacement of one or more of the hydrogen atoms in ammonia by organic radicles, such as methyl, $CH_3$—, ethyl, $C_2H_5$—, or phenyl, $C_6H_5$—, gives rise to compounds known as amines or substituted ammonias, which still retain the property of salt formation possessed by the parent substance ammonia.

For example :—



Methylamine  Methylamine hydrochloride, or Methyl ammonium chloride

Diethylamine  Diethylamine hydriodide

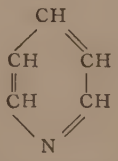


Trimethylamine  Trimethylamine hydrobromide

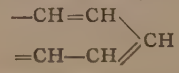
These three substances, $CH_3NH_2$, methylamine, $(C_2H_5)_2NH$, diethylamine, and $(C_2H_5)_3:N$, triethylamine, are types of three different classes of amines, known respectively as primary,

secondary, and tertiary amines, according as one, two, or three of the hydrogens of ammonia have been replaced by organic radicles.

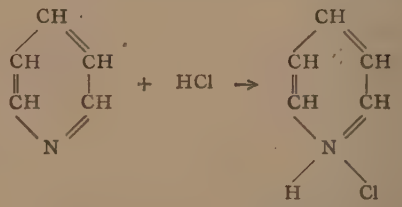
Tertiary amines are also known in which the nitrogen atom takes part in the formation of a ring, as, for example, in pyridine—



which may be regarded as being derived from ammonia by the replacement of three atoms of hydrogen by the five carbon ring—



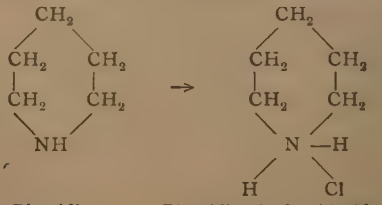
Pyridine, being a substituted ammonia, can form salts by changing the valency of its nitrogen atom from three to five, as follows :—



Pyridine — Pyridine hydrochloride

Secondary amines containing a nitrogen atom in the ring are also known.

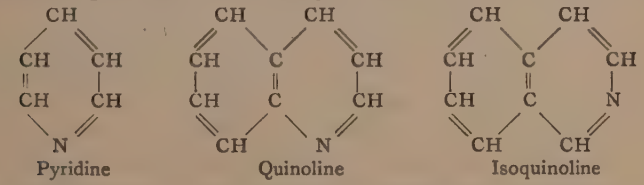
Thus, when pyridine is reduced by nascent hydrogen, six atoms of hydrogen are added on, and a substance known as piperidine is produced; this substance is a secondary amine, since it now has a hydrogen atom attached to its nitrogen. Like pyridine, it can also form a salt with hydrochloric acid.



Piperidine — Piperidine hydrochloride

From the above examples it will be seen that the presence of a trivalent nitrogen atom in a compound, whether in a ring or attached to a straight chain, will, as a rule, confer on that compound basic properties, owing to the tendency of that nitrogen to become pentavalent by combining with an acid and producing a salt. It is this property which gives rise to the term *Nitrogen base.*

The discovery and isolation from natural sources of a number of nitrogen bases, such as cinchonine, quinine, brucine, strychnine, morphine, etc., having properties analogous to those of the alkalis in being able to form salts with acids, led to their designation as alkaloids or alkali-resembling substances. As the number of such substances increased, a distinction began to be made between animal and vegetable alkaloids. The term alkaloid is, however, better reserved for nitrogen bases of vegetable origin; it was at one time suggested that the term should include only derivatives of pyridine, quinoline, and isoquinoline,




but this definition excludes such compounds as stachydrine and hygrine, etc., which are pyrrolidine derivatives, and also the purine bases which, according to most authors, should be included among the alkaloids.

This difficulty is, however, overcome by defining alkaloids as nitrogen bases of vegetable origin whose nitrogen atom forms part of a ring.

Even this definition is not entirely satisfactory, as it would include substances which, owing to their properties, could hardly be classed as alkaloids, and excludes others, such, for example, as hordenine.

## ALKALOIDS.

### *Occurrence.*

The alkaloids do not appear to have a wide distribution in the vegetable kingdom. Amongst the Angiosperms, the Apocynaceæ, Leguminosæ, Papaveraceæ, Ranunculaceæ, Rubi-

aceæ and Solanaceæ stand out in the provision of several of these substances. The Labiatæ, Rosaceæ, Orchidaceæ, and Monocotyledons and Gymnosperms very rarely contain them.

Alkaloids may occur in solution in the cell sap, especially in young parenchyma : in older tissues the substances in question may be stored in the solid state. They are found in the seeds and fruits more particularly, but in the case of the alkaloids of the Solanaceæ and some other plants they occur in the leaves, whilst the roots are the chief sources of the alkaloids of *Aconitum*, *Corydalis*, and *Hydrastis*. The cinchona alkaloids, and also pelletierine of the pomegranate, are contained in the bark of their respective trees.

With regard to their distribution in the different members of the plant, there is so much variation that a single example must serve. Stanek * found that the percentages of betaine, expressed in terms of dry weight, occurring in *Lycium barbarum*, were young leaves 3·91, old leaves 1·62, flowers without calyx 1·5, young shoots 1·55, bark of root ·49, and wood ·12.

### *Classification.*

The classification of the alkaloids is based upon the structure of the nucleus upon which their molecules are built up. Five groups of alkaloids are accordingly recognized.

I. *Pyridine Alkaloids.*—These, as the name implies, are all derivatives of pyridine, and include :—

Coniine from *Conium maculatum.*

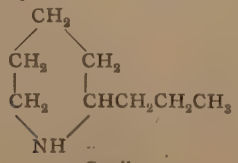
Arecolin from *Areca catechu.*

Trigonellin from *Trigonellum fœnum, Pisum sativum*, etc.

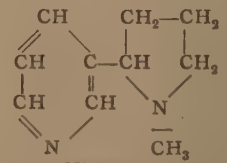
Piperine from *Piper*, and

Nicotine from *Nicotiana tabacum.*

Some idea of the structure of the molecules of alkaloids belonging to this group may be obtained from the two following constitutional formulæ, which represent coniine and nicotine respectively :—



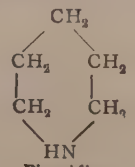
Coniine



Nicotine

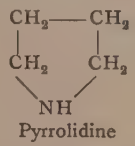
* Stanek : "Zeitsch. Zuckerind.," 1913, 37, 385.

From these formulæ it may be seen that coniine is derived from pyridine, or more strictly from piperidine—



Piperidine

whilst nicotine contains two rings, one a pyridine ring and the other a pyrrolidine ring—



Pyrrolidine

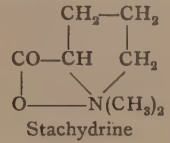
such as is also found in proline (see p. 325).

II. *Pyrrolidine Alkaloids.*—This is a small group, comprising as yet only three alkaloids, namely :—

Hygrine and Kuskhygrine, from the leaves of *Erythroxylon Coca*, and

Stachydrine, from the tubers of *Stachys tuberifera** and leaves of *Citrus vulgaris.*†

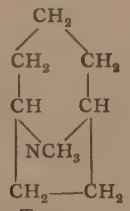
The constitution of stachydrine is as follows :—



Stachydrine

showing it to be a dimethyl betaine of pyrrolidine.

III. *Tropane Alkaloids.*—The alkaloids belonging to this group are derivatives of tropane—



Tropane

* Planta and Schulze : "Arch. d. Pharm.," 1893, 305 ; "Ber. deut. chem. Gesells.," 1893, **26**, 939 ; Schulze and Trier : *id.*, 1909, **42**, 4654 ; "Zeit. physiol. Chem.," 1910, **67**, 59.

† Jahns : "Ber. deut. chem. Gesells.," 1896, **29**, 2065.

which substance, as may be seen, contains both a six-membered piperidine ring and a five-membered pyrrolidine ring.

The group includes alkaloids from the four Natural Orders :—

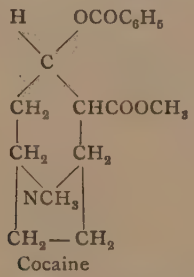
Solanaceæ, e.g. Atropine, Hyoscine, Hyoscyamine.

Erythroxylaceæ, e.g. Coca alkaloids, such as Cocaine and Tropacocaine.

Myrtaceæ : Pelletierine, Isopelletierine, etc., from *Punica granatum* (pomegranate).

Papilionaceæ: Cytisine from *Cytisus Laburnum ;* Lupinine from *Lupinus luteus* and *Lupinus niger.*

Most of the above alkaloids have a very complex constitution, and the formula of only one will be given, namely, cocaine :—




Cocaine

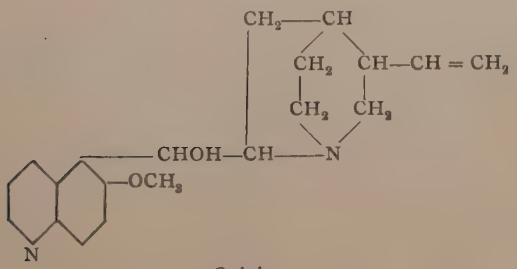
IV. *Quinoline Alkaloids.*—These fall into two groups :—

(*a*) Cinchona alkaloids, such as Quinine, Cinchonine, etc., from the bark of various species of *Cinchona* (Rubiaceæ).

(*b*) Strychnos alkaloids, such as Strychnine and Brucine from *Strychnos nux vomica, S. Ignatii,* etc., and Curarine from *Strychnos toxifera* (Loganiaceæ).

The constitution of quinine is represented by the following formula * :—

* This formula, though probably correct, has not yet been confirmed by synthesis.



Quinine

from which it will be seen to contain a quinoline ring.

The constitution of strychnine and brucine has not yet been determined, though possible formulæ have been suggested by Perkin and Robinson.*

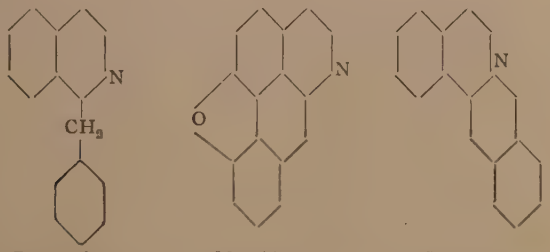
V. *Isoquinoline Alkaloids.*—These may be divided into the three following groups:—

(*a*) Papaverine group, including Papaverine, Narcotine, Laudanosine, etc., closely allied to which are Hydrastine and Hydrastinine from *Hydrastis canadensis.*

(*b*) Morphine group, including Morphine, Apomorphine, Thebaine, and Codeine.

(*c*) Berberine group, including Berberine and Corydalis Alkaloids.

The constitutional formulæ for alkaloids of this group are for the most part exceedingly complex, and it will suffice here merely to show the skeleton formulæ of a member of each group:—



Papaverine Morphine† Berberine

In addition to the alkaloids mentioned above, there are a very large number which cannot as yet be classified, since

* Perkin and Robinson: "J. Chem. Soc., Lond.," 1910, **97**, 305.
† This formula is subject to revision.

their constitution is not entirely known; these include amongst others ergotinine from ergot, colchicine from *Colchicum*, taxine from *Taxus baccata*, aconitine from *Aconitum Napellus*, delphinine from *Delphinium*, etc.

### GENERAL PROPERTIES OF ALKALOIDS.

The alkaloids are, as a rule, composed of the four elements, carbon, hydrogen, nitrogen, and oxygen, but a few are known, such as coniine, nicotine, and one or two little-known ones, such as hymenodictine and conessine (from bark of *Wrightia antidysenterica*), which contain no oxygen.

There are a few alkaloids which are liquid, e.g., coniine, nicotine, pelletierine, sparteine, etc., but by far the greater number are colourless crystalline solids. They are, as a rule, insoluble in water, but dissolve in neutral organic solvents, such as ether, amyl alcohol, chloroform, carbon tetrachloride, etc., whereas their salts have just the opposite solubilities.

They are mostly free from smell, but coniine, nicotine, and sparteine have strong odours.

Most of them have a bitter taste and are possessed of marked physiological or toxic properties.

They are all bases, and accordingly have an alkaline reaction in solution, though it must be borne in mind that aqueous solutions of the salts usually have a strongly acid reaction due to hydrolytic dissociation.

The majority of alkaloids are optically active, rotating the plane of polarized light to the left, though a few, such as coniine, laudanosine, pelletierine and pilocarpine, are dextrorotatory.

### GENERAL REACTIONS OF ALKALOIDS.

The alkaloids are precipitated from solution by a large number of different reagents with formation of amorphous or sometimes crystalline precipitates.

The commonest of these reagents are the following:—

1. A solution of iodine in potassium iodide, sometimes known as potassium ter-iodide, gives a chocolate-brown precipitate.

2. Mercuric iodide in potassium iodide,
3. Tannic acid,
4. Phosphotungstic acid,

} all of which give colourless amorphous precipitates.

5. Auric chloride,
6. Platinic chloride (see p. 276),

} which give crystalline precipitates often having characteristic melting points.

The alkaloids are, however, not the only substances which are thrown out of solution by these reagents, since most nitrogen bases behave in a similar way, and the formation of a precipitate is therefore not conclusive proof of the presence of alkaloids. On the other hand, if none of the above reagents produce precipitates, it is tolerably certain that there are no alkaloids present.

In examining plant tissues for alkaloids, Errera recommends testing the fresh sections with alkaloidal reagents and also sections which have been soaked in a five per cent alcoholic solution of tartaric acid. In the second case no precipitate should be obtained, owing to the extraction of the alkaloid.

The final identification of the various alkaloids is usually effected by means of colour reactions.

Thus, if a section of the endosperm of *Strychnos nux vomica* be mounted in a few drops of strong sulphuric acid, the presence of strychnine is indicated by a red coloration of the cell-contents. This colour will change to violet on placing a small crystal of potassium chromate beneath the cover-glass.

Similarly, a section of the rhizome of *Aconitum Napellus*, when treated with a few drops of 50 per cent sulphuric acid, will show a carmine red coloration, due to the presence of aconitine, in the parenchyma surrounding the vascular bundles. This reaction is the more marked when the section has been previously warmed in a solution of sucrose.

These colour reactions are very numerous; for them the larger text-books and monographs must be consulted.

### *Isolation.*

Most alkaloids do not occur free in the plant, but combined with some acid in the form of a salt; the acids most

commonly met with are tannic, malic, citric, succinic and oxalic, while acetic and lactic acids are rarer; some acids occur only in connexion with certain alkaloids, such as meconic acid with opium and quinic acid with quinine.

In some few cases the alkaloids can be extracted from their natural sources by means of organic solvents, such as chloroform, carbon tetrachloride, ether, etc., but in the majority of cases the alkaloid requires to be set free first by the addition of an alkali, such as lime or baryta, since only the free bases, and not the salts, are soluble in the above-mentioned solvents.

The material to be extracted is mixed with slaked lime and carefully dried, and then extracted in a Soxhlet extractor with chloroform or carbon tetrachloride; the extract is then shaken up with dilute sulphuric acid, whereby the sulphate is formed; the acid layer containing the salt in solution is then run off and evaporated, when the alkaloid salt crystallizes out and can be further purified by recrystallization.

*Example.*—Preparation of quinine from cinchona bark. Twenty grams of quicklime are stirred up with 200 c.c. of water and then thoroughly mixed in a mortar with 100 grams of cinchona bark which have been ground up in a coffee mill. The resulting mixture is then dried over a water bath, care being taken to prevent the formation of lumps. The dried substance is then extracted in a Soxhlet apparatus with chloroform. The extract is then shaken up with 25 c.c. of dilute sulphuric acid, the chloroform layer being run off from below; it is then shaken up with water several times and the water and acid extracts are mixed together and neutralized with ammonia. On evaporating the solution, quinine sulphate crystallizes out; the amount obtained rarely exceeds 1-2 grams in weight.

A rapid way of testing a piece of bark for quinine consists in heating it in a dry test tube. If there is any quinine present, the bark will give off a carmine-coloured vapour.

## THE ORIGIN OF ALKALOIDS IN THE PLANT.

Gadamer* expresses the view that the primary products of assimilation are the same for proteins and for alkaloids. When assimilation is intense alkaloids are produced, but during periods of diminished assimilation the enzyme which synthesized proteins may break down the alkaloids, the disintegration products of which may be used in the formation of proteins.

According to Pictet,† alkaloids are produced in the plant in two successive stages, involving (1) the breakdown of complex nitrogenous substances, such as protein or chlorophyll, with the production of relatively simple basic substances; (2) the condensation of these relatively simple substances with other compounds present in the plant, with the formation of the complex molecules possessed by the alkaloids.

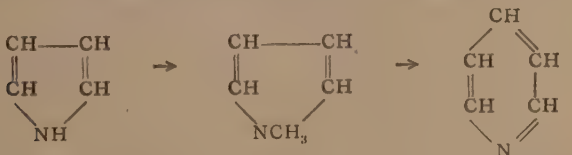
The processes of metabolism within the plant would therefore be strictly analogous to those taking place in the animal body, in which waste products, such as phenol, glycine, etc., are coupled up with other substances, such as sulphuric or benzoic acid, before being eliminated.

Pictet is further of opinion that among the commonest changes within the plant are the methylation of hydroxyl or amino groups by formaldehyde, according to the equations—

$$ROH + CH_2O = ROCH_3 + O$$

and $$RNH + CH_2O = RNCH_3 + O$$

the resulting methylated compounds being then able to undergo intramolecular transformation, by which the methyl group can enter the ring, and so produce, for example, a pyridine ring from methyl pyrrole, a reaction which he has been able to effect in the laboratory by heat.




Pyrrole Pyridine

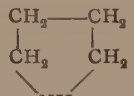
Similar changes would also explain the formation of quin-

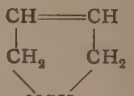
* Gadamer: "Ber. deut. Pharm. Gesells.," 1914, 24, 35.

† Pictet: "Arch. Sci. Phys. Nat.," 1905, [iv], 19, 329; "Ber. deut. chem. Gesells.," 1907, 40, 3771.

oline and isoquinoline, and it thus becomes possible to account for the origin of the pyridine and quinoline rings which occur in alkaloids, by assuming them to have been produced as above from pyrrole or indole rings, which are the normal constituents of protein (e.g. proline, histidine, tryptophane, etc.).

In support of these views, Pictet states that he was able to isolate by steam distillation from various leaves,* etc., treated with sodium carbonate, a number of simple bases which he calls proto-alkaloids; these include pyrrolidine and methyl pyrroline—


Pyrrolidine


Methyl pyrroline

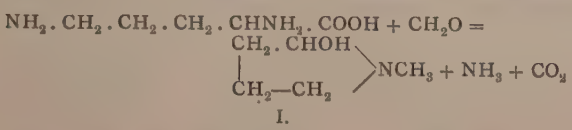
whose origin from the protein molecule is readily intelligible, in view of the fact that a similar ring occurs in proline, the cleavage product of a number of proteins. It is assumed that these proto-alkaloids are subsequently methylated, rearranged and condensed as described above to form the more complex alkaloids.

It has been suggested by Pictet that the secretion of alkaloids by plants is merely due to the inability of such plants to get rid of their nitrogenous products of metabolism by any other means than by converting them into alkaloids, which, though poisonous to animals, are not toxic to the plants themselves.

Robinson, from his work on tropinone,† offers a theory of the mechanism of the photochemical synthesis of certain alkaloids which differs fundamentally from the opinions of Pictet,‡ The raw materials—formaldehyde, ammonia, amino acids, and acetone dicarboxylic acid—for building up alkaloids either occur as such in the plant or in a combined state. These highly reactive bodies undergo a series of comparatively simple transformations ultimately leading to the alkaloid. Thus the condensation of formaldehyde with a diamino acid such as ornithine would account for the pyrrolidine group;

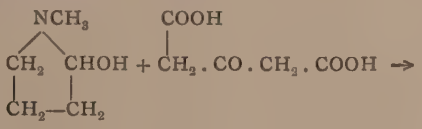
* The leaves used were those of tobacco, carrot, parsley and coco.
† Robinson: "Journ. Chem. Soc., Lond.," 1917, III, 762, 876.
‡ *Loc. cit.*

a compound of the formula I. could be formed by the interaction of these two substances according to the equation—

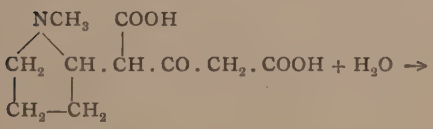


I.

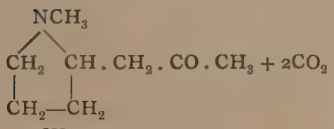
This compound would yield the alkaloid hygrine (IV.) by condensation with acetone dicarboxylic acid and subsequent elimination of carbon dioxide :—



II.



III.



IV.

Compound III. may also be the progenitor of nicotine by further condensation with formaldehyde and ammonia. Similarly, by the application of simple reactions, e.g. aldol condensations, oxidation, or dehydration Robinson is able to account for the formation of such complex alkaloids as the pelletierines, sparteine, and the opium alkaloids belonging to the piperidine, quinuclidene and isoquinoline groups respectively.

## PTOMAINES.

Associated with the simplest form of plant life, namely, bacteria, a number of different basic substances are found, some of very simple constitution, such as methylamine,

$CH_3NH_2$, dimethylamine, $(CH_3)_2NH$, trimethylamine, $(CH_3)_3N$. putrescine, $NH_2(CH_2)_4NH_2$, cadaverine, $NH_2(CH_2)_5NH_2$, and others rather more complex, such as choline, muscarine, neurine, collidine, etc., and some of unknown constitution, such as mydaleine and sepsine. These substances are known as ptomaines,* from the fact that they are usually associated with decomposing flesh; some of them, such as putrescine and cadaverine, are practically non-poisonous, while others are highly toxic, producing increased salivation, diarrhœa, vomiting, etc.

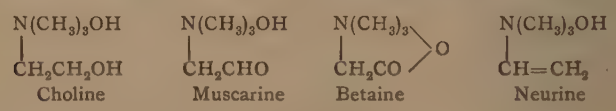
On the whole, however, it is at least doubtful whether the manifestations of ptomaine poisoning are to be attributed entirely to these substances; it would seem more likely that they were largely due to bacterial toxins, a class of substance related to the albumoses, which have the power of inducing the formation in the blood of antibodies, or, as they are better called, anti-toxins. Similar toxins or toxalbumins also occur in certain of the higher plants, as, for example, abrin, obtained from *Abrus precatorius*, and ricin, which occurs in *Ricinus*.

The so-called ptomaines are all decomposition products of the complex nitrogenous substrate upon which the moulds or bacteria are growing, but are not actually found within the organisms themselves.

In the higher forms of plant life, on the other hand, these bases are actually secreted by and stored up in the plants; the substance muscarine, for example, occurring in the fungus † *Amanita muscaria*.

Considerations of space will not permit more than the very briefest reference to the chemistry of these substances.

The compounds choline, muscarine, betaine, and neurine are closely related, as may be seen from their formulæ:—




* From the Greek word πτωμα, meaning corpse.

† The same remark applies also to the Angiosperms, which contain their alkaloids stored up in different parts of their structure.

the relationship to each other of the first three being that of alcohol, aldehyde and acid anhydride.*

Choline and muscarine occur in the toad-stool, *Amanita muscaria.* Betaine and choline frequently occur together, as for example in the germ of *Hordeum sativum*, *Triticum sativum*, *Vicia sativa*, *Lathyrus sativus*, *Gossypium herbaceum*, and several other plants. Betaine alone occurs in the juice of the beet † and in tubers of *Helianthus tuberosus.* Choline is far more widely distributed, and occurs in seeds and fruits of a very large number of plants, such as *Pinus cembra*, *Areca Catechu* (nut), *Cocos nucifera* (endosperm), *Acorus calamus* (root), *Fagus silvatica*, *Cannabis sativa* and *C. indica*, *Humulus Lupulus*, etc.

Neurine does not occur in plants, but is produced in putrefying fish and meat. Muscarine and neurine are both very poisonous, whereas choline is comparatively innocuous.

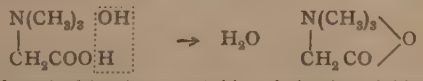
All these substances are strong bases, and answer the general reactions for alkaloids (which see).

A few other bases of comparatively simple constitution which occur in plants may here be mentioned.

Trimethylamine, $(CH_3)_3N$, is a very volatile substance which occurs in the seeds of *Mercurialis annua* and in the flowers of *Cratægus Oxyacantha*, *Pyrus Aucuparia*, and many other plants, and is given off from the leaves of *Chenopodium Vulvaria.* It is also readily produced from choline and betaine, and is, therefore, commonly produced from putrifying animal or vegetable matter containing lecithin (see p. 49).

Parahydroxyphenylethylamine, , is a substance occurring in ergot, which has a marked pressor

* The name betaine is derived from the fact that this substance was first obtained from the beetroot (*Beta vulgaris*). It is the anhydride of hydroxytrimethylamino-acetic acid.



The alkaloid stachydrine (see p. 265) is a derivative of this substance.

† For the preparation of betaine from this source, see "Ber. deut. chem. Gesells.," 1912, **45**, 2411.

action on the circulation, and causes contraction of the uterus. Its close relationship to tyrosine, from which it can be obtained by loss of carbon dioxide, is of interest.

Tyrosine — Hydroxyphenylethylamine

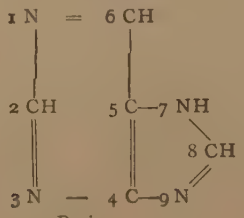
Hordenine, , is the dimethyl derivative of the previous compound, and occurs in barley.

The fact that all nitrogenous bases form crystalline derivatives with such substances as platinic or auric chlorides, or with picric or picrolonic acids is frequently made use of for isolating or identifying small quantities of these substances (see choline, lecithine, p. 49); since the derivatives produced can, as a rule, be identified by their crystalline form and melting point, they provide a certain method of recognizing substances which do not give any characteristic colour reactions.

An additional advantage of the method lies in the fact that the reagents employed (auric or platinic chloride, etc.) being substances of high molecular weight produce crystalline derivatives whose weight is very considerably greater than that of the substance which is being isolated, and thus ponderable quantities of substance may be obtained from comparatively small amounts of material.

## PURINE BASES.

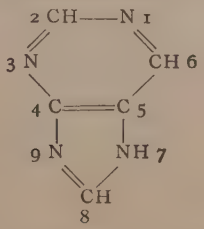
Under this heading are included such substances as caffeine, theobromine, xanthine, guanine, etc., which are called purine bases because they are all derivatives of the same substance, purine, whose formula is given below:—




Purine

This substance, which is also the mother substance of uric acid, does not occur in nature, but has been synthesized by Fischer.

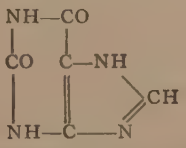
By writing the formula somewhat differently, as follows :—




it will be seen that it is composed of two rings, the upper one, which is six membered, being a so-called pyrimidine ring, while the lower one, which is five membered, is an imidazol or glyoxaline ring, the same as occurs in histidine (see p. 325).

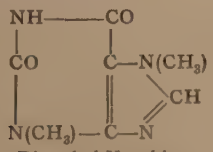
The relationship between purine, xanthine, theobromine and caffeine is best understood from the following considerations.

Xanthine may be regarded as purine with the addition of two atoms of oxygen attached to the carbon atoms numbered 2 and 6 ; and it is accordingly called 2 : 6 dioxypurine, and is given the formula :—

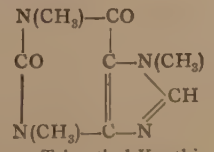



Xanthine or 2 : 6 dioxypurine

From this compound theobromine and caffeine are derived by replacing two and three atoms of hydrogen respectively by methyl groups, as may be seen from the following formulæ :—




3 : 7 Dimethyl Xanthine or Theobromine




1 : 3 : 7 Trimethyl Xanthine or Caffeine

Xanthine is widely distributed among plants, notably in

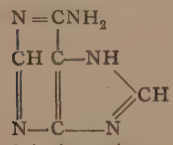
sprouting seedlings, and occurs also in tea leaves and in the juice of the beetroot.

Theobromine occurs chiefly in the fruit of *Theobroma Cacao* (1·5-2·4 per cent), and a small quantity also occurs in kola nut and in tea leaves, but not in coffee; it acts as a powerful diuretic and has a stimulating effect on the central nervous system, but is less powerful in this respect than caffeine.

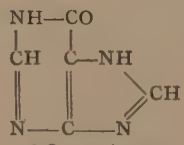
Caffeine occurs to the extent of about 1-2 per cent in kola nuts, ·1-·8 per cent in cocoa beans, from 2-5 per cent in tea leaves, from 0·8-1·7 per cent in coffee beans, and from 2·5-3 per cent in the fruit of *Paullinia cupana;* the latter substance ground up into a paste is consumed in South America under the name of guarana. The so-called Maté or Paraguay tea, the dried leaves of *Ilex paraguensis*, contains about 0·2-1·6 per cent of caffeine.

Caffeine is a powerful cerebral stimulant, but also acts somewhat on the heart; it is furthermore a powerful diuretic.

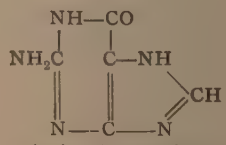
Three further purine bases deserve mention, namely, Adenine, Hypoxanthine and Guanine, the formulæ of which are as follows :—



6 Aminopurine or Adenine



6 Oxypurine or Hypoxanthine



2 Amino 6 oxypurine or Guanine

All three substances have been obtained by the hydrolysis of nucleo-proteins from plants (see p. 335) and of nucleic acids from yeast * and from *Triticum sativum*.†

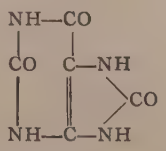
Guanine and Hypoxanthine are usually found together; they occur in sprouting seeds of a number of plants, notably *Cucurbita Pepo*, *Acer pseudoplatanus*, *Vicia sativa*, *Trifolium pratense*, *Lupinus luteus*, *Hordeum sativum*, and in the juice of the beet, etc.

Adenine, which is less widely distributed, likewise occurs in the juice of the beet and in tea leaves, and has also been found in leaves of *Trifolium repens*.

* Schittenhelm and Schröter: "Zeit. physiol. Chem.," 1904, **41**, 290.

†Osborne and Harris: *id.*, 1902, **36**, 85; Osborne: "Amer. Journ. Pharm.," 1903, **9**, 69.

Uric acid, which is systematically named 2 : 6 : 8 trioxypurine, has the formula—



It does not occur in plants, but is a well-known product of metabolism in the animal world. In view of the close relationship between this substance and the other purine bases, the assumption does not seem unwarranted that the purine bases in the plant are also waste products (see below). And, in this connexion, it is interesting to find that the presence of urea, in very small amounts, has been observed by Fosse* in the higher plants. To what extent this substance is a physiological product of the cell is doubtful.

The identification of individual members of the purine bases is not very easy, although the recognition of a purine base as such is rendered simple by the so-called murexide test which is given by practically all the members of this group of compounds.

The test consists in evaporating the substance (uric acid or caffeine may be used) in a porcelain basin with dilute nitric acid over a water bath. A yellowish residue remains which on the addition of ammonia or by exposure to ammonia vapour turns pink; potash changes the colour to purple.

The identification of caffeine in plants has been the subject of numerous researches †; it is precipitated by several alkaloidal reagents from solutions containing concentrated hydrochloric acid, but not from neutral solutions; these precipitates are, however, not characteristic. Behrens ‡ has described methods of identifying this substance with the help of mercuric chloride and of silver nitrate and nitric acid. The method is as follows:—

* Fosse: "Compt. rend.," 1912, **155**, 851; 1913, **156**, 567, 938; **157**, 948; 1914, **158**, 1374; **159**, 253; "Ann. Chim.," 1916, [ix], **6**, 13, 155. See Verschaffelt: "Pharm. Weekblad," 1914, **51**, 189, for summary of work on urea in plants.

† Clautriau: "Nature et Signification des Alcaloïdes végétaux," Brussels, 1900.

‡ Behrens: "Anleitungen z. mikrochemischen Analyse d. wichtigsten organ. Verbindungen," 1897, IV, 14.

Fifty mgs. of dried tea leaves are coarsely powdered and mixed with quicklime and sufficient water to make a crumbly mass. The mixture is then dried and extracted with alcohol; the extract is evaporated drop by drop on a microscope slide and finally the residue is sublimed by heating until it turns brown, the vapour being condensed on a second slide held about 2 mm. above it. The sublimate consists of well-formed needle-shaped crystals. A drop of water containing a trace of hydrochloric acid is then placed near the sublimate and a grain of mercuric chloride is dissolved in the drop. On drawing the mercuric chloride solution through the sublimate, colourless glistening prismatic crystals are produced.

Silver nitrate in the presence of a small quantity of nitric acid produces under similar circumstances woolly aggregates.

### PHYSIOLOGICAL SIGNIFICANCE OF NITROGEN BASES.

In considering the physiological significance of alkaloids, questions naturally arise with regard to their place in the metabolism of the plant. Are they connected with the elaboration of food? or are they so much waste material, bye-products of metabolism, corresponding to uric acid and such-like substances excreted by the higher animals? Unfortunately, definite answers are not possible; what may be true of one group of nitrogen bases may be incorrect for another, and in any case the answers would not appear to be of general application, owing to the restricted occurrence of some of these compounds in the vegetable kingdom.

Certain organisms, more especially lower ones, can use alkaloids as a raw food-material, provided they be supplied in a sufficiently dilute state; thus certain Fungi seemingly can assimilate morphia. Amongst the Algae, Comère * found that *Ulothrix subtilis* and *Spirogyra crassa*, grown under aseptic conditions and in a solution free from nitrates, could make use of certain alkaloids as a source of nitrogen. Of the alkaloids used, this was found to be true for the sulphates and hydrochlorides of atropine, cocaine and morphine; quinine, although it had no deleterious action, was not assimilated, whilst strychnine showed a marked toxic action. Clautriau † found

* Comère: "Bull. Soc. Bot., France," 1910, 57, 277.
† Clautriau: *loc. cit.*

that alkaloids supplied to the higher plant as the sole source of nitrogen are not utilized.

With regard to the higher plants, De Vries considers that alkaloids are not essential for the well-being of the plant, since in the germination of the seed of the potato, the thorn-apple (*Datura Stramonium*) and nux vomica (*Strychnos nux vomica*), little or no diminution in the substances in question occurs. This opinion is to a certain extent supported by the fact that their presence depends, at any rate in some cases, on the conditions of cultivation; for instance, quinine does not occur in cinchona cultivated in hot-houses in this country.

From the facts relating to the distribution of betaine in plants, Stanek * concludes that this substance is not a nitrogenous reserve but is used up by the plant during its development.

Lotsy † considers that alkaloids, such as quinine, are not decomposition products of proteins, but direct synthetic substances. In the case of *Cinchona*, he found that the bases occur in parenchyma cells, provided that they do not contain calcium oxalate, either in solution in the cell sap, when the tissue is very young, or in a solid state in older parts. They are first formed in the leaves, and ultimately transferred to the bark.

On the other hand, caffeine and theobromine, which strictly speaking are purines, are generally considered to be decomposition products of proteins, ‡ they are formed in places of great cellular activity and their disappearance is never accompanied by a concomitant increase of albuminous substances.

These particular substances may correspond to urea and uric acid of higher animals, for the purine nucleus is characteristic of xanthine bases, such as uric acid; and derivatives of xanthine, such as guanine and adenine, are found in caffeine and theobromine. In this connexion one important point of distinction between animals and plants may be mentioned; in the higher animals there is a definite elimination of these waste nitrogenous substances from the organism, and

* Stanek: "Zeitsch. Zuckeined.," 1913, 37, 385.
† Lotsy: "Bull. Inst. Bot. Buitenzorg," No. 3, 1900.
‡ Clautriau: *loc. cit.*

the output bears a definite relation to the amount of proteins taken as food. In plants, on the other hand, there is no general elimination of nitrogenous waste, such substances being used up in anabolic processes. Thus Weevers,* whilst recognizing that caffeine and theobromine may be the products of the decomposition of proteins, considers that they are reorganized, and are therefore not to be classed as waste products in the same sense as uric acid is. It will, of course, be noticed that there is relatively much more nitrogen in these compounds than in the proteins.

Finally, some of the substances in question may be of biological importance as a protection against herbivorous animals and parasitic fungi.

No suggestions of any real value other than those already mentioned have as yet been made concerning the source from which these substances are synthesized, although, as pointed out by Meldola,† the discovery that glucose could by the action of ammonia in the presence of zinc hydroxide be converted into methyliminazole,‡ renders the genesis of some of the natural alkaloids which contain the iminazole ring at any rate a chemical possibility.

*Weevers: "Proc. Koningkl. Akad. Wetens.," Amsterdam, 1903, 369; "Ann. Jard. Bot. Buitenzorg," 1907, **21**, 1.

†Meldola: "J. Chem. Soc., Lond.," 1906, **89**, 764.

‡Windaus and Knoop: "Ber. deut. chem. Gesells.," 1905, **38**, 1166.

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# SECTION VIII.

## THE COLLOIDAL STATE.

A KNOWLEDGE of the properties associated with the colloidal state of matter is of the greatest importance in the study of the chemical and physical problems presented by both plants and animals; for this reason some of the more important facts concerning colloids are here set forth. To illustrate the bearing of this subject on plant chemistry, it is only necessary to point out that the protoplasmic contents of any living cell exhibit many of the properties of colloidal solutions, and, indeed, it is held by some that the chief vital function of protoplasm is due to its acting as a colloidal medium.

Apart, however, from the living cell contents, many of the reserve and waste products of the vital activity of the cell are colloidal substances. Thus, for example, the cell wall itself is composed of cellulose, a substance which exhibits all the characteristic properties of colloids, while starch, resins, gums, rubber, proteins, and enzymes are all colloidal in nature. Moreover, many of the processes of dyeing and staining employed in microscopical technique are directly due to the colloidal nature both of the material to be stained and of the staining solution; further, a number of the properties of soil and humus are directly attributable to the colloidal properties of these substances.

Before considering the properties of matter in the colloidal state, it is necessary to explain the origin of the term colloid. While studying the laws of diffusion in liquids, Thomas Graham found that water soluble substances could be divided into two classes:—

(*a*) Those that diffused relatively quickly, and

(*b*) Those whose rate of diffusion was very slow or imperceptible.

The former class, including substances such as salts, acids, bases, cane sugar, urea, etc., which, for the most part crystallized readily, he called "crystalloids," while for the latter class, which comprise such substances as starch, albumen, and gum, he devised the term "colloid". Although there was this marked difference between these two classes of substance in the rates of free diffusion into pure water, it was found that the presence of a colloid, in relatively low concentration, had but little effect in retarding the rate of diffusion of a crystalloid, which accounts for the fact that diffusion experiments can be carried out in gelatine solutions (see page 300), and also that crystalloids will diffuse quite readily through colloidal membranes, such as parchment, etc.

On the other hand, it was found that such membranes offered a very strong opposition to the passage of other colloids; this observation was turned to account in the dialyser, by means of which apparatus it was found possible to separate crystalloids from colloids contained in the same solution. Numerous modifications of Graham's original apparatus have been devised, but they are all ultimately based on the same principle that if a mixed solution of a colloid and a crystalloid are separated from pure distilled water by a colloidal parchment or other membrane, the crystalloid alone will diffuse out at a measurable rate, whilst the colloid will remain behind. The method is, indeed, to this day the only one known for purifying a colloid from a crystalloid since the ordinary methods applicable for the purification of crystalloids do not hold for colloids.

The origin of the terms crystalloid and colloid was, however, based on a misconception. The rate of diffusion of any substance is in no way connected with its ability to crystallize, or the reverse since, as was subsequently shown, almost all crystalloids can be made under suitable conditions to give solutions in which they have lost their ability for rapid diffusion, and have acquired many of the characteristics of the class of substance known to Graham as colloids; similarly, many of Graham's colloids, such as egg albumen and hæmoglobin, have been obtained in crystalline form. The properties of the colloidal solutions are, therefore, no longer regarded as being due to the intrinsic properties of the substances dissolved, but

rather to the state of aggregation of the substances concerned. Only on this assumption is it possible to understand how one and the same substance can at one time produce a colloidal solution, and at another an ordinary crystalloidal solution as is, for example, the case with gallic acid, which gives a colloidal solution in water, but not in glacial acetic acid.

Graham, moreover, found that many substances which were insoluble in water in the ordinary way could, nevertheless, be made to produce colloidal solutions exhibiting the characteristic reluctance to diffuse. Since Graham's time, almost all the metals and their insoluble oxides, sulphides, carbonates, sulphates, etc., have been obtained in so-called colloidal solution, including even such insoluble substances as lead and barium sulphates.

It would appear, therefore, that the properties of a colloidal solution are not so much due to the substance itself as to the peculiar nature of the solution, or, in other words, the state of aggregation of the dissolved substance.

The evidence in support of this view is partly optical (Tyndall phenomenon, ultramicroscope, etc.) and partly direct, since it has been shown that many of the substances which are known to us as insoluble can, by a sufficient degree of disintegration, be made to yield colloidal solutions.

Thus many metals are obtained in colloidal aqueous solution by passing a powerful electric discharge between two poles of the metal held under water, and, again, a number of insoluble crystalloids, such as silica, molybdenum oxide, and vanadium oxide, have been made to yield colloidal solutions by merely finely powdering, or grinding these substances under water.*

Finally, the whole question has been shown to be amenable to mathematical treatment by Von Weimarn,† who has worked out the conditions which determine whether a given substance will assume the crystalloid or the colloidal state.

It will be seen from the foregoing that whereas in a true solution the dissolved substance is in a state of molecular dispersion this is not so in what is known as a colloidal solution,

* Wegelin: "Kolloid Zeitschr.," 1913, 14, 65.

† For an account of this see Taylor's "The Chemistry of Colloids," London, 1915.

which may be regarded as a state midway between a true solution and a suspension. The evidence of the ultramicroscope* goes to support this view.

In a true suspension the particles are of varying size, but even the smallest are visible under the magnification of a high-power microscope, the limits of visibility of which are somewhere of the order of 0·1 $\mu$, in which $\mu$ = ·001 mm. or 1 millionth part of a meter. The particles of a colloidal solution, on the other hand, may vary between the limits 0·1 $\mu$ and $\mu\mu$;† such particles, although beyond the limits of direct visibility by the microscope, can nevertheless be revealed indirectly by means of the ultramicroscope, the principle of which is to detect the presence of particles by the light reflected from them in a dark field—in much the same way as a beam of sunlight entering a dark room reveals the presence of dust particles by reflected light.

When the particles in a solution are of a smaller diameter than $\mu\mu$ they are no longer detectable by the ultramicroscope, and in a true solution they are assumed to have diameters of the order of 0·1 $\mu\mu$—the molecule of hydrogen being calculated as having a diameter of 0·16 $\mu\mu$.

It may be assumed then that in colloidal solutions we are dealing with non-homogenous mixtures or two phase systems, and that the characteristic properties of such colloidal solutions are attributable to this peculiar state of aggregation.

This explains at once why the rate of diffusion of substances in colloidal solution should be slower than those in true solution, since the larger particles would naturally be expected to move more slowly than the particles of molecular dimensions found in true solution. Moreover, it accounts for the low values obtained in the measurement of the osmotic pressure of colloids by the freezing-point method.

If osmotic pressure is ultimately caused by the impact of particles upon the walls of the containing vessel, then the more sluggish larger particles would produce fewer impacts and therefore a lower osmotic pressure than the more rapidly moving particles of molecular dimensions. Direct determina-

* For a description of this apparatus and its use see Zsigmondy: "Colloids and the Ultramicroscope". Trans. by Alexander. New York, 1909.

† $\mu\mu$ = ·001 $\mu$ = 1 millionth part of a millimeter.

tions by Starling, Lillie, Moore and Roaf, Bayliss, and others have indeed shown that colloids have a small but measurable osmotic pressure which is not due to any accidentally adhering crystalloidal impurities.

Assuming, then, that colloidal solutions are two phase systems, a phase being any particle of matter bounded by its own surface, two classes of such solutions are distinguished :—

(1) Suspensoids in which, as in a true suspension, the discontinuous or disperse phase is a solid while the continuous phase is a liquid; and

(2) Emulsoids in which, as in an emulsion, both the continuous and the disperse phases are liquid.

## SUSPENSOIDS.

Although the suspensoids are biologically of but slight importance, since only the inorganic colloidal solutions belong to this group, a brief description of their properties is essential to a survey of the whole subject.

Colloidal solutions of otherwise insoluble substances may be obtained in a variety of ways—such as electrical disintegration of the metals, reduction of metallic salts, the formation of the substance under special conditions or in particular solvents, etc., for the details of which one of the many text-books on Colloidal Chemistry may be consulted.

### GENERAL PROPERTIES OF SUSPENSOIDS.

#### A. *Optical Properties.*

Suspensoid sols, as a general rule, appear more or less clear to the unaided eye, but are frequently highly coloured. This is notably so in the case of the metallic sols such as gold and silver, which may be obtained in a variety of different shades, depending on the method of preparation and the consequent size of the particles. Thus gold sols may be either blue, purple, pink, or red, the latter containing the smallest particles, while silver sols have been obtained brownish-red, yellow, green, grey, or blue. Some of the colour effects occasionally met with in partly developed photographic plates are probably

due to absorption compounds of silver particles of various degrees of dispersion with unreduced silver chloride.*

Most suspensoid sols, however, although appearing clear when examined in the ordinary way, exhibit what is known as the Tyndall phenomenon; by this is meant the fact that when a beam of light is projected into the solution, in an otherwise dark room, the path of the beam is rendered visible by the reflection of light from the particles of the disperse phase. If examined through a Nicol prism the luminous beam is found to be polarized, which distinguishes it at once from the similar effect produced by passing a ray of light into a true solution of a fluorescent substance.

*Brownian Movement.*—In 1827, R. Brown, the botanist, first observed, by the aid of a compound lens, the peculiar movement of pollen grains suspended in water; this movement was subsequently found to be common to all sufficiently small particles similarly suspended in a liquid of low viscosity, and the phenomenon is now known as Brownian movement. The phenomenon is generally regarded as a manifestation of molecular motion, and, as is to be expected, the smaller the particles the more rapid the movement. Thus for particles of diameter 3 $\mu$ it is only a barely perceptible oscillation, but it rapidly increases with diminishing size; in the case of particles of diameter 10-50 $\mu\mu$, which are beyond the range of visibility of the microscope, Brownian movement is manifested by a rapid rectilinear zig-zag oscillation of minute spots of light in a dark field. The general motion resembles that of the flight of gnats and the velocity is of the order of 100 $\mu$ per second.

### B. *Electrical Properties.*

When a suspensoid sol in pure water free from electrolytes is subjected to the action of a powerful electric field, by dipping into it two platinum electrodes with a potential difference of about 200 volts, the sol wanders to one or other of the electrodes, showing that it bears an electrostatic charge of opposite sign to that of the electrode in question. This phenomenon is known as Kataphoresis.

The majority of suspensoid sols bear a negative charge

* Luppo, Cramer and Reindeers: "Kolloid Zeitschr.," 1911, 9, 10.

and consequently wander towards the anode; on the other hand, the metallic hydroxides, silicic acid and basic dyes, etc., wander to the cathode. While these statements are true for aqueous sols the conditions are exactly reversed when turpentine is the medium. This reversal of charge with the solvent is governed by the rule that "non-conductors in contact with a liquid assume a + or − charge according as their dielectric constant is > or < that of the liquid".

Since water has a very high dielectric constant it is natural that most other substances should assume a negative charge in relation to it.

The fact that suspensoid sols bear a recognizable electric charge renders them sensitive to electric influences, and they are consequently readily discharged by colloids of opposite sign or by electrolytes. This electrical discharge brings about a coalescing of the colloidal particles with the formation of larger aggregates and consequent precipitation resulting in the destruction of the colloidal solution. Such a change is irreversible, for the precipitate once formed cannot be redissolved.

(1) *Precipitation by Electrolytes.*—The precipitation is in this case, according to Hardy, effected by the ion of opposite sign; thus, for example, a negatively charged sol such as arsenic sulphide is precipitated by the metallic ion of an electrolyte; the precipitating power of such ions is a function of the valency and therefore in the order shown by the following series: -NaCl, $BaCl_2$, $AlCl_3$.

That the metal really enters into close relationship with the arsenic sulphide is shown by the fact that the latter when precipitated persistently retains barium hydroxide whilst the solution becomes acid due to liberation of hydrochloric acid.

The formation of a river delta by the precipitating action of sea salts upon the positively charged suspended clay particles is an illustration on a large scale of an analogous phenomenon.

Positively charged colloids, such as ferric hydroxide, on the other hand, are precipitated by the anion of an electrolyte, the precipitating power again increasing with the valency as indicated by the series sodium chloride, sulphate, citrate.

(2) *The Precipitation of Colloids by Other Colloids of Opposite Electric Sign.*—This phenomenon was first observed by Linder and Picton, who found that certain solutions of organic dyes, on mixing, produced precipitates. Further investigations have shown conclusively that only oppositely charged colloids could mutually precipitate; thus, arsenic sulphide, which is negatively charged, is not precipitated by any other negatively charged colloid, but is precipitated by ferric hydroxide, which is positive. The resulting gel is described as an adsorption compound (see below under Adsorption).

This mutual precipitation of colloids has many very important practical applications; for example, the use of ferric salts in the purification of sewage water is probably due to the precipitation of negatively charged colloidal particles of sewage by the ferric hydroxide hydrosol.

Similarly it has been suggested that the process of dyeing is really a mutual gel formation between the colloidal dye and the colloidal fibre; similarly the interaction between toxin and antitoxin, and the phenomenon of bacterial agglutination, etc., may be regarded as examples of the mutual precipitation of two colloids.

This same phenomenon can also be conveniently employed for determining the electric sign of a colloid. Thus, if a piece of filter paper is wetted, it assumes a negative charge and consequently if it is dipped into a positive dye sol the dye will be discharged on coming in contact with the paper, and water alone will be drawn up by capillary forces. If, on the other hand, the dye is a negatively charged one it will travel up the paper together with the water. This may be well shown by means of two solutions of night blue and alkali blue respectively, as recommended by Wo. Ostwald. The same principle has been worked out into a complicated system of capillary analysis by Goppelsroeder,* Freundlich,† and others.

* Goppelsroeder: "Kapillaranalyse," Basel, 1906.
† Freundlich: "Kapillarchemie," Leipsig, 1909.

## PROTECTIVE ACTION OF COLLOIDS.

The sensitiveness of suspensoid sols to electric influences can be considerably reduced by what are known as protective colloids.

Many organic substances, such as gelatine, agar, etc., when added in small quantity to inorganic colloidal solutions, can prevent the precipitation of the latter by electrolytes; under these conditions the organic colloids are said to exert a protective action upon the inorganic colloid.

It is not known in what way this protective action is exerted, but it has been suggested that the particles of the suspensoid become covered with a layer of gelatine and so acquire the properties of gelatine particles.

Suspensoids, so protected, can be evaporated to dryness, and the residue when taken up with water will redissolve.

The greatly increased stability thus acquired by the inorganic colloid makes the process of value for the preparation of colloidal solutions of the metals, particularly silver and mercury, which are used for various medicinal purposes.

A measure of protective power was first worked out by Zsigmondy,* who defined as the gold number, the number of milligrams of colloid which, when added to 10 c.c. of a bright red colloidal gold solution containing from ·0053 to ·0058 per cent of gold, is just insufficient to prevent the precipitation (as shown by the colour change to violet) of the gold by 1 c.c. of a solution of sodium chloride, containing 100 grams of salt in 900 c.c. of water.

Appended is a list of some of the commoner colloids with their corresponding gold number taken from Zsigmondy's paper:—

| Colloid. | Gold Number. | Reciprocal Gold Number. |
|---|---|---|
| Gelatine | ·005-·01 | 200-100 |
| Isinglass | ·01-·02 | 100-50 |
| Gum arabic | ·15-·25 | 6·7-4 |
| Tragacanth | 2 | 0·5 |
| Dextrin | 6-12 | ·17-·08 |
| Potato starch | 25 | ·04 |
| Mucilage from quince kernel | ∞ | 0 |

* Zsigmondy: "Zeit. anal. Chem.," 1901, 40, 697.

According to Oden * the humic acid of the soil exerts a protective action on clay, preventing its coagulation by electrolytes.

*Electric Endosmose.*—This term is applied to a phenomenon which in a sense may be regarded as the inverse of Kataphoresis. Whereas in the latter case it is the disperse phase which wanders in the electric field while the solvent, or continuous phase, remains at rest, the reverse conditions hold in the case of Endosmose. This is effected by placing the colloidal sol in a vessel the walls of which are impermeable to the colloid but permit of the free passage of the continuous phase. When this is submitted to a high difference of potential the effect is to draw the solvent out of the containing vessel and thereby to dehydrate the sol. Attempts have been made to apply this principle to the problem of the economical dehydrating of peat for the purpose of obtaining fuel but so far they have not been very successful.

It has also been proposed to dry timber by electrosmotic removal of the cell sap or to preserve the timber by replacing the sap removed by a suitable preservative. The principle of Kataphoresis has also been applied to the dehydration and purification of clay.†

## EMULSOIDS.

The Emulsoids form the second great group of colloids and from a biological point of view they are the more important of the two.

Substances such as albumen, gelatine, gums, starch, agar, etc., which belong to this group, tend to swell up in contact with water, thus indicating a tendency for close association between the substance and its solvent; for this reason the term Lyophilic colloid has been employed by some authors to these substances; the term Lyophobic being, by contrast, applied to the suspensoids.

Emulsoids are in fact regarded as consisting of a liquid disperse phase composed of a concentrated solution of the

* Oden: "J. Landw.," 1919, 67, 177.

† For a fuller account of these processes see "Second Report on Colloid Chemistry," etc., British Ass. Reports, 1918.

substance suspended in a liquid continuous phase composed of a much diluter solution. The term emulsoid, has been adopted as indicating their general relation to the emulsions which likewise are two phase systems produced from two liquids which are immiscible.

### GENERAL PROPERTIES OF EMULSOIDS.

The outstanding feature of the emulsoids as compared with suspensoids is their much greater viscosity; this fact, however, is not surprising if the views put forward with regard to their constitution are correct, since true emulsions are known to have high viscosities, e.g. Mayonnaise sauce. The viscosity of a solution varies both with the concentration and the temperature; it is liable to be influenced by a variety of causes such as prolonged heating and by different methods of treatment. In some cases the passage through a capillary tube will alter the viscosity of a solution and in some instances the viscosity will diminish spontaneously. Viscosity is, moreover, considerably affected by the presence of dissolved salts, being increased by sulphates, phosphates and citrates but reduced by iodides or sulphocyanides.

(*a*) *Optical Properties.*—These are in many respects less striking than those of the dispersoids since emulsoid sols, although frequently opalescent or turbid, are not as a rule highly coloured. The presence of the diffracting particles of the disperse phase may, however, in some cases cause a bluish opalescence as, for example, in a starch solution; indeed, according to Bancroft,* the blue colour † of eyes and feathers is caused by the same phenomenon. In common with suspensoids, the emulsoids also exhibit the Tyndall phenomenon.

Examined under the ultramicroscope they also show Brownian movement, but this is not so well defined as in the case of suspensoids; this is probably due to the fact that there is not the same difference in refractive index between the disperse and continuous phases in the case of the

* Bancroft: "J. Phys. Chem.," 1919, **23**, 356, 365.

† According to Wo. Ostwald the blueness of the sky is similarly due to the atmosphere being composed of matter in a disperse phase suspended in a continuous phase.

emulsoids since, as will be seen below, the disperse phase itself contains a considerable proportion of the dispersing medium.

According to Bayliss* the pseudopodia of *Amoeba* when examined with intense dark ground illumination show numerous minute particles in Brownian movement, which may be taken as affording evidence of the colloidal nature of the protoplasm.

(*b*) *Electrical Properties.*—Compared with suspensoids, the emulsoids are relatively stable towards electrolytes; the former are liable to be precipitated from their solutions by the merest traces of electrolytes, and hence a number of precautions have to be adopted in preparing them to exclude contamination with such bodies. The emulsoids, on the other hand, are frequently contaminated by considerable quantities of electrolytes without detriment to their solubility.

The reason for their comparative indifference to electrolytes is to be found in the absence of well defined electrical characteristics. Typical emulsoids, in fact, when pure have no electric sign, and only acquire one on the addition of either acid or alkali to their solutions. Thus, it has been shown by Hardy that whereas native albumen, when free from electrolytes, is electrically neutral, it acquires a negative charge on the addition of a little alkali, and a positive charge on the addition of acid.

According to Pauli† this accounts for the fact that positively charged metallic hydroxides are unable to precipitate electrically neutral albumen, but precipitate albumen which has become negatively charged by the addition of a little alkali; and similarly negatively charged colloids, such as phosphomolybdic or phosphotungstic acid or certain negative dyes, are only able to precipitate albumen after it has acquired a positive charge by the addition of acid.

(*c*) *Precipitation by Electrolytes.*—The precipitation of emulsoids from their solutions by electrolytes is not to be regarded as due to the electrical discharge of the disperse phase by the ionic charges, as with suspensoids. The amounts of the

* Bayliss: "Proc. Roy. Soc.," 1920.
† Pauli: "Beitr. chem. Phys. Path.," 1906, **7**, 531.

salts required for precipitation are considerable, and precipitation in this case is more probably due to a redistribution of the solvent between the emulsoid and the salt added.

Metallic salts, which precipitate emulsoids, can be arranged in three groups as follows :—

(i) Sodium, potassium, lithium, ammonium, and magnesium salts.

If not left too long in contact with these salts, the precipitated colloid can be redissolved, and the process is, therefore, reversible.

Practical application has been made of this phenomenon for separating the various types of protein. Thus, for example, if an aqueous solution containing an albumen and a globulin be mixed with an equal volume of saturated ammonium sulphate solution, the globulin, being insoluble in the resulting half-saturated ammonium sulphate, is precipitated; after filtering off the globulin, the albumen may be precipitated from the mother liquor by saturating it with ammonium sulphate.

The precipitated albumen and globulin are chemically unchanged, and can be redissolved if desired.

(ii) Calcium, barium, and strontium salts.

The process in this case is reversible immediately after precipitation, but after a very short interval it becomes irreversible.

(iii) Heavy metal salts, such as those of mercury, copper, lead, or zinc.

Here the process is irreversible, owing, no doubt, to the formation of definite chemical compounds.

The case of zinc is peculiar, inasmuch as very dilute solutions of zinc salts produce irreversible precipitation of egg albumen, whereas strong solutions may either not produce a precipitate, or else cause one already formed to dissolve.*

The anion also plays an important part in influencing the precipitating power of a given salt. By arranging the various salts of sodium in the order of decreasing precipitating power, the so-called Lyotropic series are obtained as follows :—

Citrate > tartrate > sulphate > acetate > chloride > nitrate > chlorate > bromide > iodide > sulphocyanide.

* Pauli: "Beitr. z. chem. Phys. and Path.," 1905, 6, 233, 259.

Here, again, there is no relation between precipitating power and electric charge of the ion, and the fact that citric acid comes first in the list has nothing to do with its being tribasic.

The precipitating effect of a salt appears rather to be connected with its water binding power, and it may be assumed that the presence of a citrate, tartrate, or sulphate of an alkali metal leaves less water available to the colloid.

This assumption would also explain the fact that a gelatine gel containing such salts has a higher melting point than one containing a sulphocyanide which leaves the gelatine so much water that it is reluctant to set.

On the other hand, these salts are also known to affect the compressibility of water, and their action on emulsoids may possibly be connected with this fact.

The precipitating power of the anions when combined with one of the metals of the alkaline earths is exactly the reverse of that observed when the same anions were combined with the alkali metals. Thus the precipitating power of the anions increases in the order $C_2H_3O_2 > Cl > NO_3 > Br > I > CNS$, whereas when combined with the alkali metals the inhibiting power increases in this same order.

In conformity with the above facts, Pauli,* in studying the precipitation of albumen by various salts, came to the conclusion that the precipitating power of a salt was an additive property which depended on the constituent ions (see also p. 318).

Kations, as a rule, act as precipitants for albumen, while anions tend to keep it in solution.

The precipitating power of the kations increases in the following order: Mg, $NH_4$-, K, Na, Li, while the inhibiting or solvent action of the anion increases in the following order: -$C_2H_3O_2$, -Cl, -$NO_3$, -Br, -I, -CNS.

According as the precipitating power of the kation or the inhibiting power of the anion predominates the resulting salt will either precipitate or not precipitate albumen.

The observations are given below in tabular form. As

* Pauli: "Beitr. z. chem. Phys. and Path.," 1902, 3, 225; 1903, 5, 30.
† See p. 319.

shown by the arrows, the kations and the anions are arranged in ascending order of precipitating and inhibiting power respectively. The symbols + and − respectively signify that the salt does or does not precipitate albumen, the blank spaces meaning that the salt has not been investigated.

| Kations → / Anions. ↓ | Mg | $NH_4$ | K | Na | Li |
|---|---|---|---|---|---|
| Fluoride | | + | + | + | |
| Sulphate | + | + | + | + | + |
| Phosphate | | | + | + | + |
| Citrate | | | + | + | + |
| Tartrate | | | + | + | + |
| Acetate | | − | − | + | + |
| Chloride | − | − | + | + | + |
| Nitrate | − | − | − | + | + |
| Chlorate | | | − | − | + |
| Bromide | − | − | − | − | + |
| Iodide | − | − | − | | |
| Sulphocyanide | − | − | − | − | |

From this table it may be seen that the comparatively slight precipitating power of the kations, Mg and $NH_4$-, is completely neutralized by the anions -$C_2H_3O_2$ or -Cl, while the more powerfully inhibiting anions -$NO_3$ and -$ClO_3$ are able to netralize the precipitating power of the kation K as well as that of Mg and $NH_4$-. Similarly the powerfully inhibiting anions -Br, -I and -CNS, are able to counteract the precipitating power of sodium as well.

### SWELLING OF COLLOIDS OR IMBIBITION.

Whereas a water soluble crystalloid commences to dissolve as soon as it is brought in contact with water, the same is not true for most emulsoid or lyophilic colloids. Before going into solution, these substances undergo a preliminary swelling, sometimes known as imbibition; this is accompanied by the disappearance of a certain volume of water. According to an experiment described by Hatschek, one gram of gum tragacanth covered with water in a specific gravity bottle kept under water for a week had increased in weight by 0·9 gram at the end of this period; this means that in the process of imbibition the gum had succeeded in drawing into the flask 0·9 c.c. of water. In view of the resistance which water is known to offer to compression, it is clear that enormous force must have been exerted during the process.

Direct measurement of the pressures produced during

swelling were made by the botanist, Reinke,* on *Laminaria* contained in an apparatus known as the Oedometer.

Only by the application of an opposing pressure of 41 atmospheres was he able to reduce the amount of water imbibed to one-twentieth of the amount it would normally have taken up.

*Conditions Affecting Imbibition.*—(*a*) *Temperature.*—Heat is evolved during swelling, as may be seen from the following table taken from Taylor's "Chemistry of Colloids":—

| | Cals per gram of Colloid. |
|---|---|
| Gelatine | 5·7 |
| Starch | 6·6 |
| Gum arabic | 9·0 |
| Gum tragacanth | 10·3 |

This being so, heat hinders imbibition, while cold and pressure favour it. For this reason it is best in making a solution of a colloid such as agar or gelatine to allow it to swell for some time in cold water without applying any heat.

(*b*) *Presence of Impurities.*—The swelling of colloids is very considerably increased by the presence of small quantities of either acids or alkalies. In the case of fibrin M. H. Fischer† was able to increase the normal swelling in water sixfold by the presence of 0·02N hydrochloric acid. As a practical application of this may be mentioned the beneficial effect of the addition of a small quantity of acetic acid to the water employed for swelling agar previous to making a solution.

With regard to the action of salts, it is found that anions act in the order of the Lyophilic series mentioned on p. 295.

Thus the following anions favour imbibition:—

$$CNS > I > Br > NO_3 > ClO_2 > Cl;$$

while the following inhibit:—

$$-SO_4 > \text{tartrate} > \text{citrate} > \text{acetate},$$

as do also alcohol, glucose, and cane sugar.

According to Spek‡ salts such as lithium bromide or

* Reinke: "Hanstein's bot. Abhandl.," 1879, 4, 1.

† This author considers that much of the pathological swelling in animals and man is due to an accumulation of acid in the tissues, which, as a consequence, tend to draw fluid from surrounding tissues and so swell; he would even offer the same explanation for the swelling caused by an insect's bite.

‡ Spek: "Koll. Chem. Beihefte.," 1920, 12, 1.

potassium thiocyanate, which have a strong influence on the swelling of colloids, also accelerate the rate of cell division of *Paramœcium*, while calcium chloride and sulphates, which reduce swelling, retard cell division.

Many of the epidermal tissues in plants and animals are cuticularized or otherwise hardened; this prevents their swelling when brought in contact with water, thus enabling them to maintain their shape, and it is a commonplace in histological technique to harden tissues by immersion in formaldehyde or other solutions so as to counteract and prevent this same tendency. The hardening of gelatine by means of bichromate is another example of the same principle.

*Syneresis.*—This is the name given by Graham to a phenomenon which may be regarded as the reverse of swelling. Most gels, on keeping, squeeze out a small quantity of liquid which is not pure water but a dilute solution of the colloid in question. The amount of liquid thus extended varies with the concentration of the gel, and is greater for some colloids than for others. The phenomenon can be noticed on agar culture tubes, etc., and is familiar to bacteriologists.

### GEL FORMATION.

Many colloidal solutions are able, under certain conditions, to undergo a change of state known as gel formation, in which the sol loses its liquid properties and becomes more or less rigid.

In some cases the change is reversible, meaning that by suitably altering the conditions the gel will return to a solution, and in other cases the change is irreversible.

Examples of such changes are given below:—

(*a*) *Spontaneous Precipitation.*—A silicic acid sol prepared by the addition of acid to a solution of sodium silicate will, on keeping, set spontaneously to a bluish almost transparent gel. This change is irreversible.

(*b*) *Heat Coagulation.*—This change, which may be illustrated by the coagulation of egg white in boiling water, is irreversible.

An instructive experiment, due to Hardy, consists of boiling side by side in separate beakers a fairly strong and a very dilute solution of egg white in water. The strong one

coagulates while the dilute one becomes turbid only; on the addition of a small quantity of barium chloride, however, a precipitate is produced. The explanation of this phenomenon is that, owing to the dilution of the solution, the particles of coagulated protein are too small to unite together, and therefore remain apart forming a suspensoid which is, however, precipitated by the electrolyte.

(*c*) *Coagulation by Enzymes.*—The curdling of milk by rennet is a familiar example of this type of irreversible gel formation; so also is the coagulation of pectic bodies occurring in fruit juices by the enzyme pectase with the formation of gelatinous calcium pectate.

Enzymes capable of coagulating milk also occur in many plants, such as *Lolium perenne*, *Anthriscus vulgaris*, *Geranium molle*, *Ranunculus bulbosus*, *Medicago lupulina*, *Ricinus*, *Datura*, *Pisum*, *Lupinus*, etc.

(*d*) *Gelatinization by Altering the Concentration.*—If a dilute solution of gelatine in water be concentrated until it is about 5 per cent strength it will set to a jelly on cooling to the atmospheric temperature. Solutions of agar will gelatinize at much greater dilution. The change is, in both cases, reversible, for by raising the temperature, or by adding more water, the gel goes into solution again.

A gel, however, once set will require a higher temperature to liquefy than its original setting temperature. Thus a 5 per cent gelatine gel setting at about 18° C. melts at about 26° C., while an agar solution which sets at about 35-40° C. will require to be heated to over 90° C. before it melts.

### GENERAL PROPERTIES OF GELS.

Gels partake of some of the properties of both solids and liquids. With solids they share the property of maintaining their shape and of being more or less elastic, on the other hand their compressibility is very low like that of water of which they are very largely composed.

Owing to their rigid nature they lend themselves well for experiments on diffusion, and many interesting results have been obtained. One experiment, originally due to Liesegang, consists in placing a drop of silver nitrate solution on a gelatine gel containing a dilute solution of potassium bichro-

mate; after a short time concentric rings of silver chromate are deposited around the original drop; this experiment has given rise to much experimental work with other reagents under varying conditions and there is much speculation regarding the true explanation of the phenomenon. There is, however, no doubt that the experiment illustrates the possibility of diffusion of a crystalloid such as silver nitrate in a gel.

The bringing about of such periodicity, as is exhibited by the alternating layers of deposit and clear solution in an inanimate system without variations in external conditions such as temperature changes, has an important bearing on biological and other natural problems; it would appear to offer a possible explanation of the stratification observable in agate and its possible significance in connection with the many concentric ring structures or other alternating deposits found in nature will be obvious; in illustration the formation of starch grains may be mentioned. According to Küster * many plant structures such, for example, as the banded pith of *Magnolia grandiflora*, the calcium oxalate sacs of *Ficus carica*, the regular alternation of crystal bearing zones with those containing no crystals found in the bark of the Pomegranate and the zebra-like pigmentation of the succulent leaves of *Haworthia fasciata* and *Aloe varigata* may be due to similar causes; these structures at any rate may have their origin in some analogous internal rhythmic stimulus.

The peculiar concentric growth of certain moulds resulting in structures closely resembling the Liesegang rings have been studied by Munk.†

## THE NATURE OF GELS.

As already pointed out above, emulsoids are regarded as two-phase systems in which the disperse phase is a more concentrated solution, and the continuous phase a relatively dilute one. When such a solution gives a gel, the rôles of the two phases are assumed to be changed, resulting in a sort of net-

* Küster: "Beitrage z. Entwicklungsmechanischen Anatomie d. Pflanzen, Jena," 1913.

† Munk: "Centralblatt für Backteriologie," 1912, **32**, 353 and **34**, 561; also "Biol. Centrall.," 1914, **34**, 621. See also Liesegang "Naturwissensch Wochenschr.," 1913, [xii], **25**.

or sponge-like structure, of concentrated solution representing the continuous phase, whereas the disperse phase is represented by a dilute solution filling up the interstices.

Evidence for the existence of some such sponge-like or honeycomb structure has been obtained by Hardy * in studying under the microscope the formation of a gel.

It is only by postulating some analogous structure that it is possible to understand how 1 gram of agar can cause 99 grams of water to set to a stiff jelly just as the organized cell structure of many plants enables them to maintain a rigid form while consisting of practically 90 per cent. of water.

## ADSORPTION.

The phenomenon known as the occlusion of gases is an example of the adsorption of gaseous matter by a solid surface; it is exhibited to some extent by glass and platinum, but far better by wood charcoal, owing to its large superficial area; on this fact depends the use of wood charcoal, as a deodorant or for the adsorption of the last traces of gas in the production of high vacua. It is not known in what way the adsorption is effected, but the immediate effect is to produce a concentration of gaseous molecules at the surface of contact between the solid and the gas.

To all such cases of purely surface attachment the term Adsorption is generally applied, as opposed to absorption which implies something below the surface layer.

The property of adsorption is likewise one of the most important characteristics of colloidal solutions resulting directly from their great surface development. Wo. Ostwald has calculated that if a cube of material of 1 cm. edge, presenting a total surface of 6 sq. cms., were broken up it could yield $10^{18}$ cubes of $10\mu\mu$ edge ($\equiv$ ·000001 cm.), presenting a total surface of 600 square metres. Such cubes would be approximately the size of the particles of a colloidal solution, and it will therefore be seen that a comparatively small mass of the particles in such a colloidal solution must, in the aggregate, present a very considerable surface.

It has been calculated that the total surface presented by

* Hardy: "Proc. Roy. Soc.," 1912, A., 87, 29.

the particles of a red colloidal gold solution containing 0·5 grams of gold per litre amounts to about 8 sq. metres. It is, therefore, easy to understand that with such an enormous development of surface there is the possibility for a marked manifestation of adsorption by suspensoids.

In order to appreciate the effect of such surface development it is necessary to realize that all liquids tend to reduce their surface energy to a minimum; in the case of a solution this end may be assisted by increasing the concentration at the surface of any substance which lowers the surface tension. The most active substances in producing this effect are the fatty acids, soaps, albumen, enzymes, etc., and it follows, therefore, that the surface layers will be most concentrated in aqueous solutions of these substances. Direct evidence of this may be obtained in the case of many solutions; for example, some dyes, such as methyl violet, on keeping, become so concentrated at the surface as to cover themselves with a film; the same applies to solutions of albumen. By blowing bubbles into such a solution and so increasing the surface, Ramsden* was able to remove the major portion of the dissolved substance from the solution by taking away the froth. Indeed, the tendency to froth in liquids is usually a manifestation of the greater concentration of dissolved substance at the surface with the resultant lowering of surface tension. Ramsden was further able to show that when a mixture of albumen and saponin is shaken up with water, the froth is richer in saponin since this substance lowers the surface tension of water more than does the albumen. This same phenomenon, no doubt, also explains the inactivation of some enzymes which results from mere shaking, and it has been shown that the froth of such solutions has greater activity than the rest of the liquid.

The interface between the disperse phase and the continuous phase of any colloidal solution represents a surface at which increased concentration can take place and hence the tendency for adsorption which is so characteristic a property of colloids.

The concentration of a dissolved substance upon the surface of a solid introduced into a solution may be illustrated

* Ramsden: "Z. physik. Chem.," 1904, 47, 336.

by dipping a piece of filter paper into a dilute aqueous solution of congo red; after a short time the dye will have accumulated on the surface of the paper, leaving the solution much lighter in colour.

Moreover, since congo red itself is in colloidal solution and filter paper behaves in many respects like a colloid, this experiment also illustrates the phenomenon of mutual adsorption by colloids which is the principle underlying most processes of dyeing and staining, and also enzyme actions and other processes taking place in the living organism.

In this connexion there is an interesting experiment due to Bayliss * which is designed to show that although in the process of dyeing adsorption upon the surface to be dyed may be the first step, yet chemical reaction between the dye and the fibre may follow as a second stage. The experiment consists in shaking up a blue solution of the acid of congo red with well-washed aluminium hydroxide; the latter at once adsorbs the blue colour from solution, and settles down on standing; if it is now heated, the physically adsorbed congo red acid combines with the aluminium hydroxide to form the aluminium salt, a chemical reaction which is marked by the change of colour from blue to red.

In the same way Bayliss holds that in the case of enzyme action adsorption of the substrate upon the surface of the enzyme is the first stage, and that then, in consequence of the intimate contact between the two, mass action accelerates the reaction.

It is, of course, easy to understand that if adsorption takes place so readily between colloids, such as filter paper and congo red, both of which bear negative charges in water, the phenomenon must take place still more easily between oppositely charged colloids in which the mutual electrical discharge facilitates the deposition (see p. 308).

Numerous practical applications of adsorption from solutions are known, as for example in the removal of colouring matter in the purification of cane sugar, or in the removal of fusel oil from crude spirit by filtration through charcoal.

Other substances besides charcoal, such as fuller's earth and

* Bayliss: "Z. chem. Ind. Koll.," 1908, 3, 224.

china clay, have been similarly used on account of the large surfaces which they present.

From what has been said with regard to the structure of gels and the assumption that they present a sort of network with a considerable development of internal surface, it is easy to find an explanation of the use of isinglass for clearing a turbid solution or for the fact that colouring matter may be extracted from a solution by precipitating gelatinous aluminium hydroxide in it.

The purification of sewage by means of alum followed by alkali likewise depends on the adsorption of impurities by the colloidal gelatinous aluminium hydroxide, and also upon the precipitation of colloidally dissolved impurities by the electrolyte.

The deodorizing and generally purifying effect of the soil is likewise probably due largely to the adsorption by porous or colloidal constituents of such soil.

A very striking case of selective adsorption is to be found in the power which seaweeds * have of extracting iodine from the surrounding sea water, although the amount of this element in sea water is extremely small; again, in spite of the enormous preponderance of sodium over all other metals in sea water, the plant takes up practically none of this, but takes instead potassium, which is present in much smaller quantity.

Many natural phenomena can be attributed to the same cause. For example, the power possessed by soils rich in clay or humus to retain soluble potassium salts or phosphates which would otherwise be washed away by rain.

The hydrated aluminium magnesium and sodium silicates, known as Zaeolites, which are contained in clays are colloids and they react by double decomposition with the potassium salts which may be applied as manures, and, while retaining the potash, set free a corresponding quantity of lime or soda.†

In this connexion it may be mentioned that the affinity of colloids, such as humus and clay, for certain dyes, such as methyl violet or malachite green, has been employed as a rough means of detecting or estimating the proportion of

* Cameron: "J. Biol. Chem.," 1914, **18**, 335.

† Cf. van Bemmelen: "Z. anorg. Chem.," 1900, **23**, 321.

these substances in a soil. For this purpose a quantity of the soil is shaken up with the dye solution in a cylindrical vessel; on settling, the heavier particles sink to the bottom, and a band of the dyed soil constituents is formed on the surface.

Thermodynamical considerations, coupled with experimental measurements, show the fact that true adsorption takes place according to well-defined mathematical laws which enable one to decide definitely whether a certain phenomenon is due to physical adsorption or to chemical reaction; thus, it has been found that a relatively larger amount of the total substance in solution is withdrawn from a dilute than from a strong solution.

### ENZYME ACTION OF COLLOIDS.

Associated with this enormous development of surface there is, of course, a corresponding development of surface energy,* which no doubt, in part, explains the remarkable catalytic activity exhibited by colloidal solutions of the metals.

Bredig † and his collaborators have shown that a colloidal solution of platinum containing 194 grams of metal (i.e. 1 gram atom) in 70,000,000 litres of water, or a colloidal solution of gold containing 197 grams of metal in 1,000,000 parts of water, are still able to produce a distinct accelerating influence on the decomposition of hydrogen peroxide into water and oxygen.

It has long since been known that metallic platinum, more especially the variety known as spongy platinum, when left in contact with hydrogen peroxide induces the decomposition of this substance into water and oxygen, and Berzelius,‡ as long ago as 1836, pointed out an analogy between this catalytic action of platinum and the action of an insoluble ferment, such as yeast on sugar.

This suggestion has since been borne out by a number of examples of chemical changes which could be effected equally well either by means of finely divided platinum or by a ferment, e.g. the oxidation of alcohol to acetic acid by *Myco-*

* Ostwald: "Z. physik. Chem.," 1897, **23**, 172.
† Bredig: "Anorganische Fermente," Leipzig, 1901, p. 96.
‡ Berzelius: "Jahresber.," 1836, **13**, 237.

*derma aceti*, the bleaching of indigo solution by hydrogen peroxide in presence of red blood corpuscles, the blueing of tincture of guaiacum by hydrogen peroxide in presence of red blood corpuscles, etc., all of which can also be affected by spongy platinum.

Bredig carried our knowledge of the subject a step farther; by preparing colloidal solutions of the metals and comparing their action with that of various enzymes, he traced out such a remarkable analogy between the two that he has called the colloidal metal solutions "Inorganic Ferments".

The chief points of similarity between enzymes and colloidal platinum may be summarized as follows:—

1. Both platinum hydrosol and enzymes are colloids and as such are detrimentally affected by electrolytes.

2. Both platinum hydrosol and enzymes gradually decompose spontaneously or decompose more rapidly by heating.

3. There is an optimum temperature for both colloidal platinum and for enzymes to exert their catalytic action.

4. The activity of platinum hydrosol may be stimulated by the addition of alkali until it reaches its maximum value, after which the further addition of alkali causes it to fall again. Similar stimulation of enzymes by the addition of certain substances known as Zymo-exciters have been observed in case of emulsin acting on hydrogen peroxide, and of invertase acting on cane sugar.

5. The decomposition of hydrogen peroxide whether by platinum hydrosol or by hæmase, the enzyme contained in blood, is in accordance with the laws governing a monomolecular reaction.

6. A very remarkable analogy between platinum hydrosol and the enzyme of blood is that small quantities of substances which, when added to the colloidal platinum solution, destroy its catalytic action on hydrogen peroxide, also have the same effect on the oxidase of blood. Curiously enough many of these substances are blood poisons such as sulphuretted hydrogen, hydrocyanic acid, carbon monoxide, and arseniuretted hydrogen; several other substances were also found to paralyse either the platinum solution or the enzyme.

It was further observed that platinum hydrosol when

treated with very small traces of hydrocyanic acid was temporarily poisoned but recovered after a short time; a similar effect has also been observed with enzymes. The recovery is probably due to the oxidation of the hydrocyanic acid.

It was also found that the toxic effect of the hydrocyanic acid was much greater if added directly to the platinum or gold sol than if added to a sol already containing some hydrogen peroxide. Exactly similar conditions had been previously found by Schönbein* to hold in regard to the addition of hydrocyanic acid and hydrogen peroxide to blood.

In conclusion, it should be noted that Bredig, while disclaiming any attempt to trace a fanciful connexion between the colloidal metal solutions and enzymes, emphasizes the fact that the two properties of catalytic action and colloidal nature are common to both classes of compounds and regards the colloidal metals as the simple inorganic analogues of the more complex enzymes.

One further illustration might be quoted of the chemical activity which is associated with colloidal substances presenting a large surface. A calculation based on the assumption that there are five million red blood corpuscles of diameter ·007 mm. contained in 1 c.mm. of blood reveals the striking fact that the total surface presented by the blood corpuscles contained in 5 litres of blood (the amount contained in the body of a full grown man) would be about 1875 square metres. From what has gone before it is, therefore, not surprising that these corpuscles should be endowed with special properties enabling them, in the presence of the trace of iron which they contain, to play their part in the highly complex changes involved in respiration.

* Schönbein: "Zeit. f. Biologie," 1867, 3, 144.

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# SECTION IX.

## PROTEINS.

THE term protein is applied to a large variety of bodies occurring in the animal and vegetable kingdoms, which occupy a pre-eminent position in the economy of life, owing to their being the chief constituents of protoplasm.

In the plant, proteins may occur either as solid bodies or in solution in the cell sap. They may be found in all living members; in roots, stems, leaves, sieve tubes, laticiferous tissue, etc. Reserve proteins commonly are found in the solid state, especially in seeds and in vegetative organs of propagation.

These protein bodies may be either quite amorphous or crystalline; sometimes the grains are partly amorphous and partly crystalline, as in the well-known aleurone grains of the seed of *Ricinus* (castor oil).

Protein crystals may be cubical, as in the potato, falciform as in the carpellary walls of *Gratiola officinalis*, and other shapes; they may occur quite free within the cell, as in the potato, or embedded in other bodies. These embedded crystals may be found in nuclei, e.g. in the leaves of *Melampyrum arvense* and in the ovary wall of *Campanula trachelium;* in chloroplasts, e.g. *Hedera* and *Canna;* and in amorphous protein, e.g. in the seeds of *Ricinus* and *Bertholletia.*

These last, generally known as aleurone grains, are often somewhat complicated; the grain is surrounded by a protein membrane, which is less readily soluble than the remaining amorphous protein of the matrix. Embedded in the matrix is the crystalloid, and also a globoid consisting of a double phosphate of calcium and magnesium. The crystalloids vary in shape; commonly they are hexagonal and stain brown with iodine and are readily soluble in dilute alkali. Also they

may readily be stained in fuchsin. To do this, the sections should be placed in a ·2 per cent aqueous solution of acid fuchsin for twenty-four hours, washed in running water and mounted in Canada balsam in the usual way.

Several proteins may occur in aleurone grains and may be recognized by their different solubilities in water, salt solution, alkali, and alcohol. Also, the details of the composition of these grains are not the same for all plants in which they occur; for instance, in the Pæony the matrix is soluble in water, whereas in the castor-oil plant it is insoluble in water, but soluble in a strong aqueous solution of sodium phosphate.

According to Bokorny,* globulins are the common proteins occurring in the aleurone grains and crystalloids of seeds. It should be remarked that the term aleurone grain is frequently used in a generic sense to include all non-crystalline reserve protein bodies of a more or less definite shape; they are not always of the complicated nature described above, thus in the grain of wheat they are quite simple in structure and do not contain a crystalloid nor a globoid.†

## GENERAL PROPERTIES OF PROTEINS.

Until recently, comparatively little was known with regard to the chemical nature of proteins beyond the fact that they were composed of the elements carbon, hydrogen, nitrogen, oxygen and sulphur, together with, in some cases, phosphorus and iron; their existence as a separate group of compounds therefore depended chiefly on their sharing a number of general physical and chemical properties, without regard to their constitution, concerning which little or nothing was known.

A scientific definition of proteins was first given by Panzer,‡ who classified as proteins all substances which on hydrolysis yield mono- or di-amino acids. This definition is, however, too comprehensive, as it would include amongst the proteins the group of substances described by Fischer as polypeptides.

The general physical and chemical properties which are shared by the typical unaltered proteins, such as albumins and globulins, may be summarized as follows:—

* Bokorny: "Bot. Centrbl.," 1900, **82**, 289.

† For an account of the artificial production of protein grains, see Thompson: "Bot. Gaz.," 1912, **54**, 336.

‡ Panzer: "Wiener klin. Wochenschr.," 1903, **16**, 689.

A. Physical Properties.
1. Indiffusibility. 2. Coagulation. 3. Optical activity. 4. Precipitation without change.
B. Chemical Properties. [Shared by all proteins.]
1. Precipitation reactions. 2. Colour reactions.

### A. *Physical Properties.*

1. Indiffusibility.

Unaltered or native proteins * belong to that class of bodies known as colloids (see p. 283) which are unable to diffuse through a parchment or animal membrane; it is thus frequently possible to purify a protein from salts by dialysis. The separation is, however, not quantitative, and it has, hitherto, not been found possible to remove from any protein the last traces of adhering inorganic salts, so that a perfectly pure protein, which on ignition yields no ash, has not as yet been obtained by this means.

Although all proteins are more or less colloidal in nature, they possess this property of diffusion in a varying degree; thus, for example, the albumoses and peptones, which are derived from the more complex proteins by the action of certain ferments, diffuse with comparative ease. In view, however, of the fact that these substances have never as yet been obtained in crystalline form, and that such typical colloids as oxyhæmoglobin and serumalbumin have been crystallized, the rigid distinction between colloid and crystalloid can no longer be upheld.

2. Coagulation.

All genuine or native proteins on keeping undergo a curious change known as coagulation; the nature of this change is at present not understood, but as a result of it, such proteins lose their distinctive properties of solubility, and can no longer be dissolved without first decomposing them into simpler substances, as, for example, the albumoses or peptones.

Coagulation may be brought about by (*a*) Heat, (*b*) Ferments, (*c*) Alcohol.

(*a*) The solutions of all albumins or globulins may be coagulated by heating; the temperature at which the change

* The term native protein is applied to proteins which have been isolated from the tissues by some simple process which does not involve any alteration in their original properties.

takes place is characteristic for each substance, and varies from 56° C. in the case of fibrinogen to 70-80° C. for serum albumin.* The reaction of the solution as well as the presence of dissolved salts are factors which exercise a powerful influence, a slightly acid solution being most favourable for the phenomenon, whereas an alkaline reaction may prevent coagulation entirely. According to Blum, formaldehyde is also able to prevent heat coagulation.

Heat coagulation is best effected as follows. The solution is first boiled, and from 1-3 drops of dilute acetic acid are added for each 10 c.c. of liquid, according to the amount of protein present, the liquid being boiled each time before the addition of each drop.

If the amount of salts present be small, a little 1 per cent sodium chloride should first be added, as the precipitation of small quantities of protein cannot otherwise be guaranteed.

(*b*) Some proteins are rendered insoluble by the action of certain ferments, e.g., the precipitation of casein from milk by the action of rennet on caseinogen.

(*c*) The addition of absolute alcohol to a neutral or faintly acid solution of a native protein will precipitate it from solution unchanged. If, however, it be left in contact with the alcohol for some time, the protein is rendered insoluble and is coagulated.

The solution must not be alkaline, and must contain a small quantity of neutral salts.

3. Optical activity.

The solutions of all proteins are lævo-rotatory, the amount varying from $-33{\cdot}5^{\circ}$ in the case of egg albumen to $-80^{\circ}$ in the case of casein.

4. Precipitation without change.

Certain salts, such as sodium chloride and the sulphates of sodium, magnesium and ammonium, etc., have the property of throwing proteins, except peptones, out of solution. This is, however, purely a physical phenomenon, and must be distinguished from the chemical precipitation described below,

* The coagulation temperature is not sufficiently well defined to be employed as a means of identification.

inasmuch as the proteins are precipitated unchanged, and retain all their original properties and solubilities. Absolute alcohol, also, as mentioned above, precipitates the proteins unchanged, though the precipitate must not be left in contact with the alcohol, or else it will become coagulated.

With regard to the precipitating power of these various salts, it should be mentioned that saturated ammonium sulphate will precipitate *all* proteins except peptones, and consequently a solution which on saturation with ammonium sulphate remains clear, can be regarded as free from protein.

| | | |
|---|---|---|
| Furthermore, | zinc sulphate is approximately equivalent to | ammonium sulphate. |
| | saturated sodium chloride is approximately equivalent to | saturated magnesium sulphate, or<br>1/2 saturated ammonium sulphate. |

In view of the number of proteins in the plant and their different characteristic solubilities, it is easy to see the importance to the well-being of the plant of factors which have a bearing on these properties. Thus any cause which removes water, not immediately replaceable, from the cell, and so leads to a concentration of the cell sap, may be a determining factor in the existence of a plant. Cold is one such factor;* a fall in the temperature may cause the water to crystallize, so that the salt solutions in the cell become stronger, with the result that some of the proteins of the protoplasm may be dissolved and other proteins in solution may be precipitated. The importance of soluble carbohydrates and of oils in the cell sap in this connexion has already been pointed out.

It is unnecessary to remark that this effect of cold must vary pretty considerably in different plants, and depends upon the nature of the salts dissolved in the cell sap and the proteins upon which they can act. To take a few examples: it was found that in *Begonia*, soluble proteins were precipitated when the temperature reached $-3^{\circ}$ C.; on the other hand, in the leaves of *Pinus*, a temperature of $-40^{\circ}$ C. was required to obtain a similar result.† This may, in part, be due to the paucity of crystalloids in the cell sap, for it is stated that

* See Blackman: "New Phytol.," 1909, **8**, 354.
† Gorke: "Landwirth. Versuchs. Stat.," 1906, **65**, 149.

plants which are subject to periodic drought possess only small amounts of soluble crystalloids in the cell sap.

In the case of the barley, it was observed that an exposure for one night to a temperature of $-7°$ C. reduced the yield of soluble proteins by about one-third as compared with a control experiment in which the temperature was not so lowered. This salting out effect is much increased if the cell sap becomes acid on cooling, as is not infrequently the case.

If the low temperature be long continued, the precipitated proteins will not again enter into solution when the amount of water is increased by raising the temperature; on the other hand, if the temperature be suddenly raised, the precipitated proteins will re-dissolve, provided that they have not stood too long, and thus the plant will not be greatly harmed.

## B. *Chemical Properties.*

1. Precipitation reactions.

The proteins have both acid and basic properties; thus, casein may be looked upon as typically acid, seeing that it dissolves in alkalis to form sodium and potassium salts, whilst the histones and protamines are powerful bases. All proteins, however, have basic properties, which enable them to form insoluble salts with a great many of the ordinary alkaloidal reagents, such as phosphotungstic, tannic, picric, ferrocyanic, and trichloro-acetic acids. Pittom,* however, finds that many of the simpler polypeptides are not precipitated by phosphotugstic acid. They are also precipitated by potassium teriodide (a solution of iodine in potassium iodide) and by the double iodides of potassium with mercury, bismuth, and cadmium. The strong mineral acids also precipitate proteins. In consequence of this dual nature of proteins they are classed as amphoteric electrolytes (see below).

The salts of the heavy metals also produce insoluble precipitates with the proteins, a fact which is made use of in the administration of egg albumen as an antidote in cases of poison with salts of the metals. Moreover, the antiseptic action of mercuric chloride is most probably connected with this formation of insoluble salts.

Amongst the salts most frequently used as precipitants for

* Pittom: "Biochem. Journ.," 1914, 8, 157.

proteins are the chloride and acetate of iron, the sulphate and acetate of copper, the chloride of mercury and the acetates of lead and zinc.

2. Colour reactions.

These reactions depend on the fact that certain groups or radicles in the protein molecule produce characteristic colours with suitable reagents. The reactions may also be employed for detecting these same groups in the decomposition products of the proteins, with the object of determining how far the decomposition has gone, and whether it has been sufficiently deep-seated to destroy this grouping or not. The following is a list of the more important colour reactions :—

(i) *Biuret Reaction.*—This is the bluish-violet colour produced by adding copper sulphate to an alkaline solution of a protein. Unchanged proteins give a bluish-red, whilst altered proteins, such as the peptones, give a pink.

The colour is given by the substance biuret itself, whose composition is expressed by the formula $NH_2CO.NH.CONH_2$, and by similarly constituted compounds containing two —CO . NH— groups connected together through a carbon, nitrogen, or sulphur atom.

(ii) *Millon's Reaction.*—A solution of mercuric nitrate containing nitrous acid added to a solution of a protein produces a precipitate which turns pink or red. This reaction is connected with the phenolic group of the tyrosine complex in the protein molecule; it may also be used as a test for tyrosine.

(iii) *Xanthoproteic Reaction.*—Protein solutions treated with concentrated nitric acid develop a yellow colour which is intensified by heating, and is changed to orange by ammonia. This reaction is likewise connected with the tyrosine complex.

(iv) *Adamkiewicz's Reaction.*—The addition of concentrated sulphuric acid to a solution of a protein dissolved in acetic acid produces a reddish-green or violet colour. This reaction is characteristic of the tryptophane group, and is produced by the interaction of this with glyoxylic acid contained in the acetic acid; the reaction may be intensified by replacing acetic by glyoxylic acid in the test.

(v) *Liebermann's Reaction.*—Proteins, which have been previously extracted with alcohol and ether to remove fats, on warming with concentrated hydrochloric acid, develop a

violet colour. According to Cole,* the colour is due to the presence of glyoxylic acid as an impurity in the ether.

(vi) *Molisch's Reaction* is a reaction for furfurol produced by the action of concentrated sulphuric acid on a carbohydrate. The substance to be tested is treated with a few drops of a 10 per cent alcoholic solution of *a*-naphthol; concentrated sulphuric acid is then slowly added, when a red-violet colour is formed at the junction of the two liquids.

### *Microchemical Reactions.*

The following are the usual microchemical tests employed for the indication of proteins within the plant:—

1. Iodine gives a yellow to brown coloration.

2. With osmic acid a brown coloration results.

3. Biuret reaction.—A solution of copper hydrate in caustic potash may be added direct to the preparation; or the section may be steeped for some time, say twenty to sixty minutes, in ·2 per cent solution of potash, washed, placed in a 10 per cent solution of copper sulphate for thirty to sixty minutes, washed in water and mounted in a 2 per cent solution of caustic potash. A mauve to violet coloration indicates the presence of proteins.

4. Millon's reagent.—The section or scraping is mounted in a few drops of the reagent and warmed. A brick-red coloration results when proteins are present. The reagent may be prepared by dissolving some mercury in twice its weight of nitric acid (sp. gr. 1·42), the operation being performed in a fume cupboard. When the action has ceased, the solution is diluted with twice its volume of water.

5. Xanthoproteic reaction.—A yellow to orange coloration results with proteins. The preparation is warmed on the slip with a few drops of strong nitric acid. The proteins acquire a yellow colour which is changed to orange on moistening with strong ammonia.

## PROTEINS AS COLLOIDS.

An important difference between the solution of an electrolyte and of a colloid lies in the fact that whereas the electrolyte breaks up into two oppositely charged ions, the colloid appears

* Cole: "Journ. Physiol.," 1904, 30, 311.

to be charged as a whole either positively or negatively, and accordingly when such a solution is subjected to a difference of potential, the colloidal particles wander bodily to one or other of the electrodes.

Biltz has shown that two colloids mutually precipitate each other only if they bear unlike charges, and when once precipitated, they become electrically neutral and are no longer transported by an electric current. It has further been shown that only those crystalloids which are electrolytes are able to precipitate colloids, such substances as urea or cane sugar being unable to effect precipitation.

According to Hardy and Bredig, the particles in a colloidal solution are held in suspension by the opposing forces of capillary attraction and of electrostatic repulsion such as must exist between particles all of which bear the same charge; the precipitation of a colloid by the addition of an electrolyte is accordingly attributed to the elimination of the electrostatic repulsion by the fact of the charge borne by the ions of the electrolyte neutralizing the charge borne by the particles. Billitzer, on the other hand, holds the view that on introducing the electrolyte into the solution the particles tend to congregate around the ions, and are thereby brought into such close contact with each other that they form sufficiently large aggregates to be precipitated.

By means of conductivity experiments, Pauli* was able to show that pure egg albumen, free from electrolytes by repeated dialysis, was electrically neutral, for, on subjecting a solution of this substance to an electric current for twenty-four hours, no particles of albumen were transferred to either of the two electrodes. He found, moreover, that the addition of neutral salts of the alkali metals or the metals of the alkaline earths, produced no change in the electrical state of the albumen, whereas on adding traces of acids or acid salts the albumen assumed a positive charge, while on the addition of bases or salts having an alkaline reaction, it became electronegative. This observation that an electrolyte, such as hydrochloric acid, which contains an equal number of oppositely charged ions, is able to impart a positive charge to electric-

*Pauli: "Hofmeister's Beiträge," 1902, III, 225; 1904, V, 27; 1905, VI, 233, etc.

ally neutral albumen, is explained by assuming that albumen exerts some selective action on the ions, and is more permeable to positively charged hydrogen ions than it is to the negative chlorine ions. Pauli has further shown that his electrically neutral albumen, unlike ordinary albumen, is not precipitated from solution by the addition of salts of copper, iron, zinc, lead or mercury. The fact that neutral albumen is, however, precipitated by alcohol, although both substances are electrically neutral, must not be taken as evidence against the view that precipitation is produced as a result of the neutralization of the charges borne by colloidal particles; the explanation of the precipitation in this case lies in the complete insolubility of albumen in alcohol.

These facts throw some light on the electrical behaviour of native albumen in the living organism, for inasmuch as salts of the metals at once precipitate such albumen, whereas they refuse to precipitate neutral albumen, it follows that native albumen must bear a negative charge. This negative charge is most probably produced by the hydroxyl ions * liberated from the salts in contact with it, a view which receives support from the fact that on adding sodium bicarbonate to a fresh solution of neutral albumen, the latter at once assumes a negative charge.

As a consequence of this negative charge, it follows that the greater the electro-positive nature of an element, the greater will be its tendency to precipitate native albumen; on the other hand, the electro-negative acid radicles will tend to prevent precipitation, a tendency which is found to increase in the following order—sulphate acetate, chloride, nitrate, bromide, iodide and sulphocyanide.

The antagonistic action of electro-positive and electro-negative radicles accounts for the fact that sodium in the form of sodium sulphate is a precipitant, whereas in the form of sodium bromide it is not; in the case of sodium iodide and sulphocyanide, the influence of the electro-negative radicle entirely outweighs that of the electro-positive sodium, with the result that these salts not merely do not precipitate albumen themselves, but actually interfere with the precipitation of albumen by other salts.

* Pauli: "Hofmeister's Beiträge," 1906, 7, 531.

INCREASING TENDENCY TO PREVENT PRECIPITATION OF ELECTRO-NEGATIVE ALBUMEN.

| | $-SO_4$ | $-C_2H_3O_2$ | $-Cl$ | $-NO_3$ | $-Br$ | $-I$ | $-CNS$ |
|---|---|---|---|---|---|---|---|
| Li . . | + | | + | + | + | | |
| Na . . | + | + | + | + | − | − | − |
| K . . | + | + | + | − | − | − | − |
| $NH_4$ . . | + | − | − | − | − | − | − |

The sign + indicates that the reagent precipitates albumen, and − that it does not.

If, however, we make an albumen solution electro-positive by the addition of a little acid, the electro-negative or acid radicles now become the precipitants, and the whole order is reversed, and those salts which like the bromide, iodide or sulphocyanide, tended to prevent precipitation, now become the most powerful precipitants.

## AMPHOTERIC ELECTROLYTES.

Amphoteric electrolytes are defined by Bredig* as substances which in aqueous solution are able to exhibit both acid and basic properties, and are accordingly able to split off or combine with $\overset{+}{H}$ or $\overset{-}{OH}$ ions.

Thus, in the presence of bases they behave as acids and dissociate as follows:—

$$ROH \rightleftharpoons \overset{-}{RO} + \overset{+}{H}$$

whereas in the presence of acids they behave like bases, giving the following ions:—

$$ROH \rightleftharpoons \overset{+}{R} + \overset{-}{OH}$$

This phenomenon of changing electrolytic distribution according to circumstances is known as "electrolytic tautomerism". Examples of amphoteric electrolytes in inorganic compounds are to be found amongst the hydroxyl derivatives of most of the elements from the middle of the periodic table,

* Bredig: "Zeit. Electrochem.," 1899, 33.

such as aluminium, chromium, zinc, lead, tin, manganese, arsenic or antimony, all of which are weak bases or acids.

The fact that egg albumen is able to neutralize hydrochloric acid was first observed by Sjöqvist,* and later by Bugarsky and Liebermann;† moreover, the fact that both basic dyes such as rosanilines, as well as acid dyes such as picric acid, are able to combine with wool fibres, points to the amphoteric nature of the protein in this case also.

The simultaneous possession by a body of both acidic and basic groupings ‡ is well illustrated by the amino acids; the true amphoteric character is, however, only illustrated by those of them that are sufficiently weak acids, or whose acidic and basic functions are about equally strong.

The observation that the hydrochloride of albumen is precipitated by the addition of sodium phosphomolybdate, whereas albumen itself is not, led Spiro and Pemsel § to conclude that albumen belongs to a class of compounds which, although electrically charged, are not ionized, and while not functioning as acids or bases themselves, are none the less able to form addition compounds with such substances. According to Sjöqvist ‖ and Bugarsky and Liebermann,¶ albumen forms with acids and bases true salts, which obey all the laws of Van't Hoff and Arrhenius, though, on the other hand, its low conductivity would appear to preclude the possibility of its possessing well-marked hydrogen or hydroxyl ions. These facts are, however, readily explained by assuming proteins to be pseudo-bases of the type described by Hantzsch ** and his collaborators. Hantzsch describes as pseudo-bases a class of compounds which are chemically indifferent, but which on coming in contact with acids undergo molecular rearrangement, giving rise to true bases.

This change, which may be represented as follows,

$$N \equiv ROH \rightarrow R \equiv N \cdot OH$$

implies a change from a neutral carbinol to a true ammonium

* Sjöqvist: "Skand. Arch. Physiol.," 1895, **5**, 354.
† Bugarsky and Liebermann: Pflüger's "Archiv Physiol.," 1898, **72**, 68.
‡ Winkelblech: "Zeit. physikal Chem.," 1901, **36**, 551.
§ Spiro and Pemsel: "Zeit. physiol. Chem.," 1898, **26**, 270.
‖ Sjöqvist: "Skand. Arch. Physiol.," 1895, **5**, 277.
¶ Bugarsky and Liebermann: "Zeit. gesam. Physiol.," 1898, **72**, 68.
** Hantzsch: "Ber. deut. chem. Gesells.," 1899, **32**, 575, 3109; 1900, **33**, 278.

base with pentavalent nitrogen, which is able to react with acids to form salts of the type of $R{\equiv}N{\cdot}Cl$.

This assumption explains the reason why albumen is not precipitated by sodium phosphomolybdate until a little acid has been added, for if albumen in neutral solution is a pseudo-base, it would only be converted into a true base capable of being precipitated by sodium phosphomolybdate after the addition of acid.

For a further account of the connexion between pseudo-acids or bases and amphoteric electrolytes, see Zadwidzki* and Hantzsch.†

### THE DECOMPOSITION PRODUCTS OF THE PROTEINS.

The most direct way of obtaining an insight into the probable groups or groupings which occur in the molecule of some complex substance, is to break it up into simpler ones, whose constitution is already known, or may be determined with comparative ease. This is the method which has been employed to elucidate the very complex structure of the proteins.

Various processes have been employed for breaking down the protein molecule, such as acid hydrolysis, fusion with alkalis, the action of enzymes or putrefactive bacteria, oxidation, etc. As a result of all these various methods, a number of simple compounds have been obtained, which fall primarily into two main groups:—

1. *Biuretic derivatives*, such as albumoses, peptones, etc., which are still very complex substances, but have, at any rate, a lower molecular weight than the original unaltered protein. These substances all give the Biuret reaction.

2. *Abiuretic derivatives.*—In this group of cleavage products, which give no Biuret reaction, are included the various amino acids.

By an amino acid is meant an acid in which one or more of the hydrogen atoms other than the carboxylic hydrogen are replaced by the amino group $-NH_2$. Thus acetic acid $CH_3COOH$ gives rise to the amino acid known as glycine $CH_2NH_2COOH$. Theoretically it should be possible to re-

* Zadwidzki: "Ber. deut. chem. Gesells.," 1903, **36**, 3325; 1904, **37**, 153.
† Hantzsch: "Ber. deut. chem. Gesells.," 1906, **39**, 3149.

place two or even three atoms of hydrogen in acetic acid by the $—NH_2$ group to produce diamino acetic acid $CH(NH_2)_2COOH$ and triamino acetic acid $C(NH_2)_3COOH$; these compounds are, however, not known, and appear to be incapable of existing.

The next homologue after acetic acid, namely, propionic acid $CH_3CH_2COOH$, can give two mono-amino acids $CH_3CHNH_2COOH$ and $CH_2NH_2CH_2COOH$ known respectively as $\alpha$- and $\beta$- amino propionic acids, according as the amino group is attached to the $\alpha$- carbon atom, adjacent to the carboxyl group, or to the $\beta$- carbon atom, which is next but one from the carboxyl.

In the case of the higher homologues, diamino acids are known which have two amino groups attached to different carbon atoms, such, for example, as $\alpha$- $\delta$- diamino valeric acid $CH_2NH_2CH_2CH_2CHNH_2COOH$ derived from valeric acid $CH_3CH_2CH_2CH_2COOH$.

The dicarboxylic acids also can give rise to amino derivatives such as aspartic acid $COOHCH_2CHNH_2COOH$ derived from the dicarboxylic acid succinic acid $COOHCH_2CH_2COOH$ and glutamic acid $COOHCH_2CH_2CHNH_2COOH$ derived from glutaric acid $COOHCH_2CH_2CH_2COOH$.

It is important to note that all amino acids which are known to take part in the building up of the protein molecule are $\alpha$-substituted acids, as will be seen from the list of protein cleavage products given below.

The presence of the $—NH_2$ group in amino acids confers upon these substances basic properties, in addition to the acid properties which they already possess. Thus, for example, glycine $CH_2NH_2COOH$ is able to react with hydrochloric acid to produce glycine hydrochloride $CH_2NH_2HClCOOH$, just as ammonia reacts with hydrochloric acid to give a hydrochloride; on the other hand, being an acid, it is also able to form metallic salts, such as $CH_2NH_2COOK$. It is not surprising to learn that the mono-amino acids, such as glycine and its homologues, have no very pronounced acidic or basic properties; they belong, in fact, to the class of bodies known as amphoteric electrolytes (see p. 320). On the other hand, the mono-amino derivatives of the dicarboxylic acids, namely, aspartic acid $COOHCH_2CHNH_2COOH$ and glutamic acid $COOHCH_2$

$CH_2CHNH_2COOH$, are strong acids, owing to the predominating influence of the two carboxyl groups, while the diamino derivatives of the monocarboxylic acids, such as lysine $CH_2NH_2CH_2CH_2CH_2CHNH_2COOH$, ornithine $CH_2NH_2CH_2CH_2CHNH_2COOH$, etc., have strongly marked basic characteristics, the two amino groups here overpowering the single carboxyl group.

A class of substances which have to be carefully distinguished from the amino acids are the *acid amides*. These are derived from carboxylic acids by replacing the hydroxyl group of the carboxyl by $—NH_2$. Thus acetic acid $CH_3COOH$ gives the amide $CH_3CONH_2$ known as acetamide, while aspartic acid $COOHCH_2CHNH_2COOH$ gives the amide $CONH_2CH_2CHNH_2COOH$ known as asparagine.

AMINO ACIDS OBTAINED AS CLEAVAGE PRODUCTS OF PROTEINS.

(1) *Aliphatic Compounds.*

Mono-carboxylic mono-amino acids.

- Glycine or α-amino-acetic acid $CH_2NH_2COOH$
- Alanine or α-amino-propionic acid $CH_3CHNH_2COOH$
- Amino-butyric acid $CH_3CH_2CHNH_2COOH$
- Amino-caproic acid $CH_3CH_2CH_2CH_2CHNH_2COOH$
- Valine or α-amino-isovaleric acid

$$\begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!\rangle CH \cdot CHNH_2COOH$$

- Leucine or α-amino-isocaproic acid

$$\begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!\rangle CHCH_2CHNH_2COOH$$

- Isoleucine or α-amino-β-methyl β-ethyl propionic acid

$$\begin{matrix} CH_3 \\ C_2H_5 \end{matrix}\!\!\rangle CHCHNH_2COOH$$

- Serine or α-amino β-hydroxy propionic acid $CH_2OHCHNH_2COOH$

Dicarboxylic mono-amino acids.

- Aspartic * or α-amino-succinic acid $COOH\,CH_2CHNH_2COOH$
- Glutamic * or α-amino-glutaric acid $COOH\,CH_2CH_2CHNH_2COOH$

* The amides corresponding to these two acids, namely asparagine $CONH_2CH_2CHNH_2COOH$ and glutamine $CONH_2CH_2CH_2CHNH_2COOH$ are of considerable importance in plants. The former occurs in asparagus and is produced in seeds which are allowed to germinate in the dark (Schulze, "Landwirtsch. Gahrb.," 1878, 411), while the latter has been found in the seeds of *Cucurbita* and many other plants (Schulze and Barbieri, "Ber. deut. chem. Gesells.," 1877, **10**, 199; Schulze, *id.*, 1896, **29**, 1882). Asparagine and glutamine being readily hydrolysed by mineral acids, are not obtained as cleavage products of proteins by the ordinary methods of chemical hydrolysis, and for this reason are not quoted in the above list of cleavage products.

Mono-carboxylic di-amino acids.

Ornithine or α-δ-di-amino-valeric acid

$NH_2CH_2CH_2CH_2CHNH_2COOH$

Arginine or δ-guanidine α-amino-valeric acid

$HN=C(NH_2)NH\,CH_2CH_2CH_2CHNH_2COOH$

Lysine or α-ε-di-amino-caproic acid

$NH_2CH_2CH_2CH_2CH_2CHNH_2COOH$

Di-amino-trihydroxy-dodecanic acid * $C_{12}H_{26}O_5N_2$

Dicarboxylic di-amino acid.

Cystine or di [β-thio α-amino propionic] acid

$CH_2(NH_2)CHCOOH—S—S—CH_2(NH_2)CHCOOH$

## (2) *Aromatic Compounds.*

Mono-carboxylic mono-amino acids.

Phenyl alanine or β-phenyl α-amino propionic acid

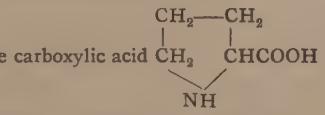
$C_6H_5CH_2CHNH_2COOH$

Tyrosine or β-parahydroxyphenyl α-amino-propionic acid

$HOC_6H_4CH_2CHNH_2COOH$

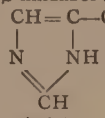
## (3) *Heterocyclic Compounds.*

Proline or α-pyrrolidine carboxylic acid

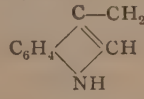


Hydroxyproline or hydroxy α-pyrrolidine carboxylic acid

Histidine or β-imidazol α-amino propionic acid



Tryptophane β-indole α-amino propionic acid



The above list comprises most of the more important cleavage products of proteins, the constitution of which has been definitely established.

Since different proteins give rise to different amounts of these various substances, it is obvious that a careful quantitative determination of the amounts of these acids produced by the hydrolysis of different proteins must be of considerable value.

To this end Fischer, in 1901, introduced his so-called

* The constitutional formula of this substance has not yet been determined.

"Ester method," which consisted in converting the mixed amino acids obtained by hydrolysis of proteins into their corresponding esters, and then separating these by fractional distillation.

The method * is best illustrated by an example. Casein was decomposed by hydrolysis with concentrated hydrochloric acid, the hydrochloride of glutamic acid being separated by filtration. The filtrate was then evaporated under reduced pressure, taken up with alcohol and saturated with dry gaseous hydrogen chloride; in order to remove the water formed by the reaction, the solution was once more evaporated down, and the residue taken up with alcohol and again saturated with hydrogen chloride. The esters were next liberated from their hydrochlorides by evaporating the solution down to a syrup in a vacuum, diluting with water and approximately neutralizing by means of strong caustic soda solution while keeping thoroughly cooled in a freezing mixture. Concentrated potassium carbonate was now added, and the esters of aspartic and glutamic acid were extracted by ether; after adding more 33 per cent caustic soda and potassium carbonate and extracting again with ether, the combined extracts were dried with anhydrous sodium sulphate, evaporated and distilled under 8-15 mm. pressure. The various fractions were then separately hydrolysed, either by boiling with water or by warming them on the water bath with 20 per cent baryta water.

This method, with slight modifications, has been applied by several workers, more especially Abderhalden and Osborne, to a considerable number of different proteins, with the result that there are now more or less reliable data for comparing the composition of proteins from various sources, both animal and vegetable.

Dakin † has suggested a method of separating the products of the acid hydrolysis of proteins by extracting with such solvents as butyl and ethyl alcohols.

A second method for gaining some insight into the composition of proteins consists in studying the distribution of nitrogen in the molecule with a view to ascertaining whether

* Fischer: "Zeit. physiol. Chem.," 1901, 33, 151.
† Dakin: "Biochem. Journ.," 1918, 12, 290.

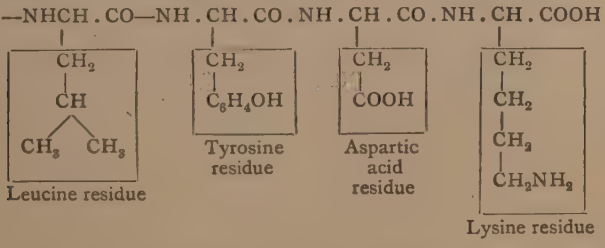
it is present in the form of mono- or di-amino acids, etc. A method for distinguishing between the different types of nitrogen-linking occurring in the molecule was first suggested by Hausmann,* and has since been modified by Gümbel; † it depends on the fact that di-amino acids, in virtue of their strongly basic character, are precipitated from solution by the addition of phosphotungstic acid, whereas mono-amino acids are not.

The substance to be examined is first hydrolysed by boiling with concentrated hydrochloric acid for several hours under a reflux condenser. The amount of amide nitrogen and ammonia in the resulting mixture is then determined by distillation with magnesia in vacuo at 40° C.

2. The di-amino acid nitrogen is next determined by precipitating the residue in the flask with excess ‡ of phosphotungstic acid and estimating the amount of nitrogen in the precipitate by Kjeldahl's method.

3. The nitrogen combined as mono-amino acids may be determined directly in the filtrate or by the difference between the total nitrogen and the sum of the nitrogens separately determined by the above methods.

The fact that proteins on hydrolysis yield such a large number of amino acids, all of which have the amino group attached to the $\alpha$-carbon atom (i.e., the carbon atom adjacent to the carboxyl), has led to the conclusion that the protein molecule is really composed of a long chain of these acids linked together in some such way as is represented below.



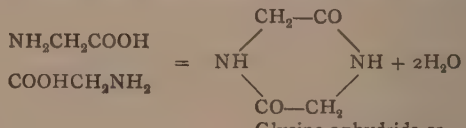

Such a compound would, of course, give the biuret reaction and contain but few free carboxyl groups or amino groups, which is entirely in agreement with the properties of proteins. Acting on this assumption, Fischer has synthesized a number

* Hausmann: "Zeit. physiol. Chem.," 1899, **27**, 95; 1900, **29**, 136.
† Gümbel: "Beitr. chem. Phys. u. Path.," 1904, **5**, 297.
‡ In order to ensure complete precipitation of arginine.

of compounds containing such a structure, with the object of studying their properties and comparing them, if possible, with natural proteins. To these synthetic substances he has given the general name of Polypeptides.

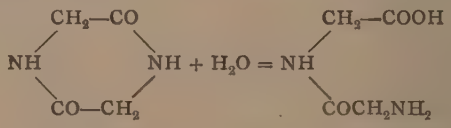
The simplest polypeptide known is glycylglycine; this substance was obtained as follows:—

Glycine, when kept for some time in aqueous solution, loses water from two molecules, giving an anhydride




Glycine anhydride or diketopiperazine

This substance, when boiled with hydrochloric acid, is hydrolysed, the ring being opened with the formation of the dipeptide glycylglycine.




To give anything like a complete account of the methods employed in the synthesis of polypeptides is outside the province of this book. It may, however, be mentioned that a very fruitful method of synthesizing these substances consists in acting on an amino acid or a polypeptide with chloracetyl chloride, thus:—

$$CH_2ClCOCl + NH_2CH_2CONHCH_2COOC_2H_5 = CH_2ClCONHCH_2CONHCH_2COOC_2H_5 + HCl$$

The latter, after conversion into the acid, and treatment with ammonia, yields a tripeptide,

$$CH_2ClCONHCH_2CONHCH_2COOH + NH_3 = CH_2NH_2CONHCH_2CONHCH_2COOH + HCl$$

Diglycylglycine a Tripeptide

Another valuable method consists in treating an amino acid suspended in acetyl chloride with phosphorus pentachloride and so obtaining an acid chloride $R_1CHNH_2COCl$. This latter is then allowed to act upon the amino group of a second acid as follows:—

$$R_1CHNH_2COCl + NH_2\underset{\displaystyle R_2}{\underset{|}{C}}H\cdot COOH = R_1CHNH_2CONH\cdot\underset{\displaystyle R_2}{\underset{|}{C}}HCOOH + HCl$$

The resulting polypeptide may, of course, be of considerable complexity, according to the nature of $R_1$ and $R_2$.

By these and similar methods, employing other combinations of amino acids, polypeptides containing a great many different groupings have been synthesized. The one with the longest chain as yet obtained is an octodecapeptide leucyltriglycyl-leucyltriglycyl-leucyloctoglycyl-glycine of the formula—

$$NH_2CHC_4H_9CO[NHCH_2CO]_3NHCHC_4H_9CO[NHCH_2CO]_3NHCHC_4H_9CO[NHCH_2CO]_8$$
$$NHCH_2COOH$$

The more complex of these polypeptides resemble the proteins in being colloidal substances which give the biuret reaction, and in being precipitated from solution by phosphotungstic or tannic acids and by ammonium sulphate.

The action of digestive ferments upon them has been studied by Abderhalden and others; they are not readily attacked by pepsin, but are hydrolysed by pancreatic or intestinal juice.

A striking confirmation of Fischer's view concerning the close connexion existing between the polypeptides and the natural proteins is to be found in the fact that the hydrolysis of proteins, under suitable conditions, has yielded four substances which could be identified with synthetic polypeptides. Thus, a solution of silk fibroin in hydrochloric acid was allowed to stand for several days; on evaporating a residue was obtained which, when digested with trypsin, yielded a peptone-like substance; the latter on hydrolysis with barium hydroxide gave glycylalanine, which was identified by its naphthaline sulphonic acid derivative.* Subsequently, the hydrolysis was repeated under somewhat altered conditions, with the same result that glycylalanine was obtained.† In a later communication, the same authors described the isolation of glycyltyrosine from the products of hydrolysis of silk fibroin, and of glycyl-leucine from elastin. Levene and Beatty ‡ also claim to have obtained prolyl-glycine from the hydrolysis of gelatine.

Abderhalden § also mentions certain substances of a polypeptide nature which he found amongst the products of pancreatic digestion of a number of proteins such as caseine, edestine, hæmoglobin, serum globulin, egg albumin and fibroin.

* Fischer and Abderhalden: "Ber. deut. chem. Gesells.," 1906, **39**, 752.
† Fischer and Abderhalden: "Ber. deut. chem. Gesells.," 1906, **39**, 2315.
‡ Levene and Beatty: "Ber. deut. chem. Gesells.," 1906, **39**, 2060.
§ Abderhalden: "Zeit. physiol. Chem.," 1905, **44**, 28, 33.

OCCURRENCE OF AMINO ACIDS IN PLANTS.

*Leucine* occurs as such in the buds of the horse-chestnut * and many other plants. Isoleucine has been discovered by Felix Ehrlich † in the residual molasses obtained from sugar refineries.

*Lysine* and *histidine* have been isolated from sprouting plants by Schulze.‡

*Arginine* has been observed in the cotyledons of lupin seeds and in etiolated pumpkin seeds,§ and also in several species of conifers.

*Phenyl alanine* was discovered by Schulze and Barbieri ‖ in etiolated germinating lupin seeds.

*Tyrosine*, according to Shibata,¶ occurs in considerable quantity in rapidly growing shoots of Japanese bamboos, and in small quantity in seedlings of *Lupinus albus* ** and *Vicia sativa*; †† it has also been described as occurring with asparagine in the root-tubers of *Dahlia variabilis*. According to Bertel,‡‡ tyrosine is converted into homogentisic acid by an oxidase (see p. 361) contained in the plant, which is a change similar to the one produced in the human body in the condition known as alkaptonuria.§§ Schulze and Castoro, ‖‖ however, deny the accuracy of these observations.

*Tryptophane* has been found in seedlings of *Lupinus albus*, *Vicia sativa*, and in *Pisum sativum*.¶¶

*Proline* is obtained by the hydrolysis of a number of proteins of vegetable origin, notably the prolamins, but has not so far been found to occur as such in any plants.

* Schulze and Barbieri: "J. prakt. Chem.," 1882, **15**, 145; Schulze and Winterstein: "Z. physiol. Chem.," 1902, **35**, 299.

† Ehrlich: "Ber. deut. chem. Gesells.," 1904, **37**, 1809.

‡ Schulze: "Z. physiol. Chem.," 1899, **28**, 465.

§ Schulze and Steiger: "Z. physiol. Chem.," 1887, **11**, 43; "Ber. deut. chem. Gesells.," 1886, **19**, 1177.

‖ Schulze and Barbieri: "Ber. deut. chem. Gesells.," 1881, **14**, 1785.

¶ Shibata: "J. Coll. Sci. Tokyo," 1900, **13**, 329.

** Schulze and Castoro: "Z. physiol. Chem.," 1906, **48**, 387, 396.

†† Gorup Besanez: "Ber. deut. chem. Gesells.," 1877, **10**, 781.

‡‡ Bertel: "Ber. deut. bot. Gesells.," 1902, **20**, 454.

§§ Wolkow and Baumann: "Z. physiol. Chem.," 1891, **15**, 266.

‖‖ Schulze and Castoro: *loc. cit.*

¶¶ Schulze and Winterstein: "Z. physiol. Chem.," 1910, **65**, 431.

### CLASSIFICATION OF PROTEINS.

The classification of the proteins was originally, for want of chemical knowledge, based on their different physical properties, such as solubilities, coagulation by heat, precipitation by neutral salts, etc.

Now that, from a study of their products of hydrolysis, a little more is known of the chemistry of the proteins, it is found that, on the whole, the physical method of classification is more or less in accordance with the chemical evidence.

Appended is the scheme of classification generally adopted in this country :—

*Protamines.*—These are the simplest proteins known, and are represented by such substances as salmine, sturine, cyclopterine, etc., which have been isolated from fish sperm.*

They usually occur associated with nucleic acid in the form of salts.

No compounds resembling the protamines have as yet been isolated from plants, although they may possibly occur in pollen.

*Histones.*—The histones, of which the best known one is that obtained from blood corpuscles, are characterized by being precipitated from solution by ammonia ; they are related to the protamines, but are more complex than these substances.

*Albumins.*—This group includes egg-albumin, serum-albumin, and such vegetable albumins as legumelin of the pea and leucosin of wheat and other cereals.

The albumins are typically *soluble* in water and are coagulated by heat. They are *not* precipitated by saturation with sodium chloride or magnesium sulphate, nor by *half* saturation with ammonium sulphate, but, like all proteins, are precipitated by complete saturation with ammonium sulphate.

Traces of albumins occur in practically all seeds, but no seeds, so far examined, have been found to contain large quantities.

While plant albumins resemble those of animal origin

* Kossel: "Bull. soc. chim., Paris," 1903, [23], **29**, I-XVIII.

in regard to the two essential features of this group, namely, solubility in water and coagulation by heat, they differ in regard to their behaviour towards strong solutions of inorganic salts. Thus animal albumins are not supposed to be precipitated by half saturation with ammonium sulphate or saturation with sodium chloride or magnesium sulphate, but this is not always found to be the case for vegetable proteins, many of which are precipitated under these conditions.

*Globulins.*—These are exemplified by serum globulin, fibrinogen, and myosinogen, and also the derivatives of the two latter, fibrin and myosin. Examples of vegetable globulins are furnished by conglutin from the seeds of *Lupinus*, edestin from the seeds of *Cannabis sativa*, excelsin from the seeds of *Bertholletia excelsa*, legumin from the seeds of *Pisum sativum*, *Vicia Faba*, and other Leguminosæ, juglansin from the seeds of *Juglans spp.*, vicilin from the seeds of *Pisum sativum*, *Vicia Faba*, etc., and vignin from the seeds of *Vigna sinensis*. In brief, globulins are amongst the commonest protein reserves of the higher plants.

The typical globulins are *insoluble* in pure water and are coagulated by heat. They are soluble in dilute salt solutions, but are insoluble in stronger salt solutions; thus, unlike the albumins, they are precipitated by saturation with magnesium sulphate or by only half saturation with ammonium sulphate.*

The vegetable globulins, which form the major portion of the reserve proteins of all seeds except cereals, do not always conform to these conditions of solubility. Thus, whereas animal globulins are insoluble in water and are precipitated by half saturation with ammonium sulphate, a great many globulins from plants are precipitated at less than half saturation, and, on the other hand, some are not precipitated until the solution is almost saturated with

* These differences in solubilities between albumins and globulins may be illustrated by dissolving some of the white of an egg in water and placing it in a dialyser; as the small quantity of sodium chloride contained in the egg-white diffuses out, the globulin is precipitated out of solution; or again, if the solution is mixed with an equal volume of saturated ammonium sulphate solution, the globulin will likewise be precipitated out, owing to the solution now being half saturated with ammonium sulphate, but the albumin will remain in solution.

ammonium sulphate. It must, however, be noted that globulins extracted from seeds are nearly always obtained in the form of salts with a small amount of acid, and so long as they are in this form they have the characteristic solubilities of animal globulins. As soon, however, as the acid is removed they lose these and become completely soluble in water.

A further point of difference between animal and vegetable globulins is that many of the latter are only coagulated by heat with considerable difficulty.

The albumins and globulins are the only classes of proteins which are coagulated with heat.

*Glutelins.*—This is a small class represented by two proteins, both of vegetable origin, namely, glutenin found in wheat and oryzenin in rice. Similar substances probably occur in other cereals as well, but owing to the difficulty of obtaining them in a pure condition, they have not as yet been investigated.

Glutelins are insoluble in water and neutral saline solutions, but dissolve in dilute alkali or acid.

*Gliadins or Prolamins.*—These also are represented only by vegetable proteins, namely, gliadin from wheat or rye, hordein from barley, and zein from wheat or maize. Up to the present they have only been found to occur in cereals. The gliadins differ from all other proteins in being soluble in 70-90 per cent alcohol, the solutions being unaltered by boiling; they are insoluble in water or in salt solutions, but are soluble in dilute acids or alkalis.

On hydrolysis they yield a considerable quantity of proline (hence the name prolamins), glutamic acid and ammonia, but only small amounts of arginine and histidine, and no lysine.

Glutelins and gliadins are the chief protein constituents of the substance known as gluten.

*Sclero-proteins.*—This term is synonymous with the older term albuminoid, and includes substances of skeletal origin, such as keratin from hair, horn, etc., gelatin, elastin, and silk fibroin.

No representative of this class has as yet been found among vegetable proteins.

*Phospho-proteins.*—This group, which is probably not represented in the vegetable world, contains such substances as caseinogen and vitellin, obtained from milk and egg yolk respectively. The phosphorus of these proteins is in intimate organic combination with the protein molecule, and is not contained in the "prosthetic group" (see below) as in the case of the nucleo-proteins, which are composed of proteins with the phosphorus-containing nucleic acids.

The phospho-proteins are insoluble in water, but soluble in alkalis.

Caseinogen and vitellin were formerly known as nucleo-albumins, but the term is misleading, owing to the confusion arising with the nucleo-proteins, which are conjugated proteins (see below), and the term nucleo-albumin has for that reason been abolished.

The phospho-proteins resemble the nucleo-proteins in their solubilities, but they differ from them in their behaviour on hydrolysis; they yield at first a so-called pseudo- or para-nuclein, corresponding to the formation of a nuclein from a nucleo-protein, but whereas a nuclein on further hydrolysis yields nucleic acid, and ultimately purine bases, the pseudonuclein yields no corresponding pseudonucleic acid, but on the other hand is broken up by baryta water into phosphoric acid, but *gives no purine bases.*

*Conjugated Proteins.*—This group may be divided into three sub-groups.

1. Chromo-proteins, represented by hæmoglobin.

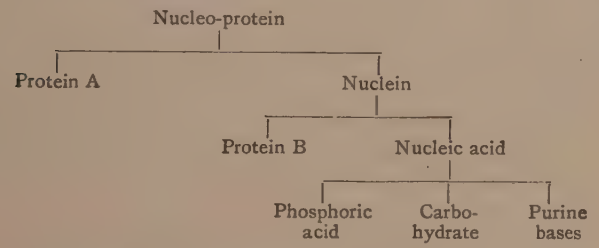
2. Nucleo-proteins, obtained from blood, chyle and lymph.

3. Gluco-proteins, represented by mucin.

Conjugated proteins are characterized by the fact that on hydrolysis they break up, yielding a true protein and a substance of a different nature, for which Kossel has proposed the name "prosthetic" group.

Thus, for example, a chromo-protein like hæmoglobin breaks up into globin (a protein) and a pyrrole derivative, hæmatin (cf. chlorophyll, p. 234). Similarly, a gluco-protein such as mucin yields a protein and a carbohy-

drate, glucosamine.* And lastly, a nucleo-protein, when subjected to peptic digestion, or treated with dilute acid, gives a protein and a nuclein; this latter with caustic alkali breaks up still further into a second protein and a nucleic acid; the nucleic acids † on further hydrolysis yield phosphoric acid, a carbohydrate residue, either a pentose or glucose, and purine bases, such as guanine, adenine, xanthine, etc. These changes are rendered clearer by the following scheme:—




It must be borne in mind that the protamines and histones frequently occur loosely combined with nucleic acids in the form of salts, but this type of combination is different from that between a protein and a nuclein such as is found in true nucleo-proteins.

The conjugated proteins appear to be rarely found in plants.

With regard to the occurrence of nucleo-proteins among plants, it is undoubtedly true that nucleic acid has been repeatedly found in plants, and compounds of proteins with nucleic acid have been isolated by Osborne, but it is not certain whether these substances actually occurred pre-formed in the seed, or were produced during the process of their isolation. Osborne‡ is

* Glucosamine is a peculiar nitrogen containing sugar of the formula

$CH_2OHCHOHCHOHCHOHCHNH_2CHO$ or $CH_2OHCHOHCH\cdot CHOH\cdot CHNH_2CHOH$ (with O bridging the first and last carbons)

It has all the ordinary reactions of sugars as regards reduction of Fehling's solution, reaction with phenylhydrazine, etc., but is not fermentable by yeast. Owing to the presence of the amino group, it is also able to form salts with acids such as hydrochloric acid. It was first obtained by the hydrolysis of chitin contained in the shell of lobsters and has since been obtained by the hydrolysis of several gluco-proteins such as serum mucoid, etc.

† For a fuller account of these compounds see Jones: "Nucleic Acids," London, 1914, and Levene: "J. Amer. Chem. Soc.," 1917, 39, 828, and 1919, 40, 415.

‡ Osborne: "The Vegetable Proteins," London, 1909.

of opinion that "only small quantities of nucleo-protein occur in the entire seed, and that this will be found chiefly in the tissues of the embryo in which the nuclei of the cells are far more abundant than in the tissues of the endosperm".

With regard to chromo-proteins and gluco-proteins, the former possibly may be represented by phycoerythrin (p. 256) and the latter by the mucilage which occurs in the roots of *Dioscorea japonica*, which in many of its characters resembles mucin from animal sources.

*Derivatives of proteins.*—In this group are included a number of substances obtained by the hydrolysis of proteins; they may be sub-divided as follows:—

1. Meta-proteins, consisting of acid albumin and alkali albumin, produced respectively by the action of acid or alkali on proteins.

2. Proteoses, represented by albumose, globulose, gelatose, etc. These substances are produced from proteins by the action of digestive juices such as pepsin and trypsin.

Pepsin, which acts in an acid medium, breaks up the protein as follows:—

PROTEIN.
META-PROTEIN (acid albumin).
PRIMARY PROTEOSE (precipitated by half-saturated ammonium sulphate and by potassium ferrocyanide in the presence of acetic acid).
SECONDARY PROTEOSE (precipitated by saturated ammonium sulphate, but only slowly by potassium ferrocyanide in the presence of acetic acid)
PEPTONE (not precipitated by saturated ammonium sulphate nor by potassium ferrocyanide in the presence of acetic acid).
POLYPEPTIDES AND AMINO ACIDS.

The formation of amino acids from peptones takes place only after prolonged action.

Trypsin, which acts in an alkaline medium, produces substantially the same series of changes, only that the meta-protein in this case is alkali albumin; furthermore the decomposition into amino acids takes place more rapidly than with pepsin.

A great many seeds have been found to contain proteoses after the removal of the other proteins, and substances resembling the proto- hetero- and deutero-

proteoses obtained from animal proteins have been described; but in all cases it is difficult to say whether these substances were not produced by some secondary action of enzymes upon the protein, during the process of isolation.

3. Peptones. Substances belonging to this class still give the biuret reaction, but unlike all other proteins they are not precipitated from solution by saturation with ammonium sulphate.

4. Polypeptides, which include such substances as leucyl glutamic acid, obtained by Fischer and Abderhalden from gliadin by hydrolysis with 70 per cent sulphuric acid, and glycyl tyrosine and glycyl leucine, obtained by the same authors from silk fibroin and elastin respectively.

### COMPARISON BETWEEN VEGETABLE AND ANIMAL PROTEINS.

From the foregoing it will be seen that, in the main, the animal and vegetable proteins conform sufficiently well with regard to their general properties and solubilities that they may be included in the same scheme of classification. The greatest irregularities are exhibited in the groups of albumins and globulins, but even these are not sufficiently serious to suggest any fundamental difference between the proteins derived from animal and vegetable sources. These views are confirmed by chemical evidence: with the single exception of di-amino trihydroxy-dodecanic acid, a substance as yet only obtained from casein, all the known products of hydrolysis of animal proteins have been obtained from vegetable proteins, and there is no real reason for assuming that there is any fundamental difference in the structure of the protein molecule from the two sources.

On the whole, vegetable proteins yield more glutamic acid, and many also yield rather more proline, arginine and ammonia than do animal proteins.

The comparatively large quantities of proline and arginine which occur in some cases may be responsible for the slightly higher nitrogen content which characterizes proteins of vegetable origin.

Further, it should be noted that the prolamins, or alco-

hol soluble proteins, form a distinct class; they are found only in the vegetable kingdom, and have no analogues amongst proteins from animal sources.

Of twenty-three different seed-proteins which have so far been systematically hydrolysed, all were found to contain leucine, proline, phenylalanine, asparagine, glutamic acid, tyrosine, histidine, arginine and ammonia; two gave no glycine; two gave no alanine; four gave no lysine; and one gave no tryptophane. One, namely zaein, gave neither glycine, lysine nor tryptophane. Three gave no cystine, and two others only traces.

It is, on the whole, unlikely that there is any protein entirely free from sulphur, although in the case of vicilin the amount is actually as low as 0·1 per cent. If it is assumed that the sulphur is contained in the molecule in the form of cystine, it follows that there must be at least two atoms of sulphur present. Calculations based on this assumption give a value for the molecular weight of at least 15,000, but although from other considerations the molecular weight of proteins is known to be high, it is unlikely that the value is as high as this.

While it is possible by means of general reactions to place a given protein in the class of albumins or globulins, there are no distinctive chemical or physical methods by which the identity of any particular albumin or globulin may be established; thus it not infrequently occurs that two substances which have been obtained from different sources, and are described under different names, are eventually found to give the same figures on analysis, and are therefore regarded as identical. This is notably the case with albumins obtained from different plant seeds, and the serum albumin derived from different animals. Within the last few years, however, a biological method has been discovered which promises to become of the very greatest value in distinguishing the various compounds from each other. Following upon the researches of Wassermann and Uhlenhuth, Tstistowitch found that serum drawn from a rabbit, which had been inoculated for some time with the serum of a horse, had acquired the property of producing a precipitate when added to normal horse serum; this is due to the formation in the rabbit's blood of a substance known as a precipitin, which

belongs to a class of compounds described by Hofmeister as pseudo-globulins; the precipitate formed is a compound of the precipitin with the albumin contained in the serum to which the precipitin was added. The precipitin so prepared should only react with horse serum and not with the serum of any other animal; the reaction is, however, not absolutely specific, inasmuch as a precipitin *may* react with the serum of an animal closely related to the one from whose serum it was prepared. It has, moreover, been found that substances which had been isolated from natural fluids, as well as native sera, were able to incite a precipitin formation when injected into the blood of some living animal, and it has been thus possible to show that the albumin contained in milk is not identical with that obtained from blood. The method has been employed by Kowarski and Schütze * for distinguishing the various plant albumins, and by Rickmann,† Uhlenhuth,‡ and others for distinguishing between horse flesh and the meat of other animals. An attempt has also been made to employ the same principle for the estimation of proteins by a comparison of the precipitates formed under various conditions.§

As illustrating the very close connexion existing between albumins and globulins, it is worthy of note that Moll claims to have converted serum albumin into serum globulin by warming a 3 per cent solution of serum albumin for one hour to 60° C. with N/66 sodium carbonate, but it is difficult to say whether true serum globulin was actually produced. According to Chick and Martin, ‖ however, the conversion of albumin into globulin may be explained merely by assuming a difference in the state of aggregation.

### EXTRACTION OF PROTEINS.

The main facts relating to the solubilities of the common vegetable proteins are as follows:—

* Kowarski and Schütze: "Deut. med. Wochenschr.," 1901, **27**, 442; 1902, **28**, 804.

† Rickmann: "Chem. Zeit.," 1907, **2**, 1983.

‡ Uhlenhuth and Weidanz: "Praktische Anleitung z. Ausführung des biolog. Eiweissdifferenzierungsverfahren," Jena, 1909.

§ Schulz: "Deut. med. Wochenschr.," 1906, **32**, no. 26; "Zeit. Unters. Nahr. u. Genussm.," 1906, **12**, 257.

‖ Chick and Martin: "Journ. Physiol.," 1912, **45**, 261.

1. Proteoses, albumins, and some globulins are soluble in water.

2. Globulins, together with most of the proteins soluble in water, dissolve in 10 per cent sodium chloride.

3. Prolamins are soluble in alcohol (70 to 90 per cent).

4. Glutelins and prolamins dissolve in dilute acid and in dilute alkali.

These facts are made use of in the extraction of the substances in question from vegetable tissues such as seeds, which may contain several proteins; and although the products so obtained are anything but pure, a brief outline of the method may be given. The separation of the proteins removed by these means from the seed by a given solvent is a very lengthy and tiresome process, and the details must be sought for elsewhere.*

Before proceeding with the extraction, the material must be ground up as finely as possible, in order that all the cells may be broken; if needs be, the tissue must be carefully dried beforehand, but too high a temperature must not be used.

In all cases the initial procedure is much the same; the main point to be observed, as in everything else, is thoroughness. The powdered material is well mixed with the solvent, which is allowed to act for some time; the mixture should be well shaken periodically. The solid is then filtered off and well washed with fresh solvent, and is again treated until the extract gives no protein reaction. The temperature may be raised during the extraction, but it should not be high enough to alter the proteins. If the extraction, especially with aqueous solvents, be prolonged, it may be necessary to add a little antiseptic, such as chloroform, in order to stop bacterial action.

When it is desired to make successive extracts, in cases such as seeds where several proteins may be present, the order may be water, 10 per cent sodium chloride, alkali (·1 to ·2 per cent caustic potash or ·5 to 1 per cent sodium carbonate), and finally alcohol (70 to 80 per cent).

The initial extraction may be made with salt solution, the albumins being afterwards separated from the globulins, and

* See Osborne: "The Vegetable Proteins," London, 1909, on whose account the following is based. For a method for the preparation of plant globulins, see Reeves: "Biochem. Journ.," 1915, 9, 508.

this course is recommended when both are present on account of the saving of time.

The proteins isolated by these means may be roughly purified as follows :—

1. Albumins and globulins.—These will nearly always be contaminated one with the other. A separation may be effected in the following ways :—

(*a*) The solution is saturated with magnesium sulphate, whereby the globulins are precipitated and the albumins remain in solution (but see above, under albumins and globulins).

(*b*) Dialysis. The extract is placed in a dialyser and floated in water which is continually changed. The precipitated globulins are filtered off from the salt solution, which, of course, is getting weaker and weaker and contains the albumins. The precipitated globulins are re-dissolved in warm saline solution, which may on cooling deposit globulins in a crystalline form ; if this does not occur, the solution may be saturated with magnesium sulphate. The albumins may be precipitated by saturating the solution with ammonium sulphate. Further purification may be effected by a repetition of the process and by fractional precipitation with magnesium sulphate or by ammonium sulphate, according to the protein to be purified.

2 Glutelins.—The proteins soluble in dilute alkali may be precipitated by very carefully neutralizing the solution and then further adding only just sufficient acid to cause the precipitation of the glutelins. The precipitate may be well washed with a dilute neutral saline solution, in which it is insoluble, in order to remove any globulins which may be present.

3. Prolamins.—The extract, which is made by treatment with hot alcohol, is either mixed with water sufficient in amount to precipitate the proteins, or the filtered solution may be evaporated under a reduced pressure at a temperature not higher than 50° C. The precipitate is filtered off and may be re-dissolved in as little alcohol as possible. From this solution the protein may be recovered by the addition of *absolute*

alcohol, in which prolamins are insoluble, and ether. The ether is added in order to make the precipitation more complete and also to hold any fats which may have been extracted by the alcohol.

### SYNTHESIS OF AMINO ACIDS IN THE PLANT.

With regard to the synthesis of animo acids within the plant, it is of interest to note that in the laboratory Erlenmeyer and Kunlin * have been able to synthesize the acetyl and formyl derivatives respectively of alanine and glycine by the action of ammonia on glyoxylic acid, both of which substances are known to occur in plants. The changes involved may be represented by the following formulæ :—

$$2\ \underset{\text{Glyoxylic acid}}{\begin{matrix}CHO\\|\\COOH\end{matrix}} + NH_3 = \underset{\text{Formylglycine}}{\begin{matrix}CH_2NHCHO\\|\\COOH\end{matrix}} + H_2O + CO_2$$

$$\begin{matrix}CH_2NHCHO\\|\\COOH\end{matrix} + H_2O \rightarrow \underset{\text{Glycine}}{\begin{matrix}CH_2NH_2\\|\\COOH\end{matrix}} + HCOOH$$

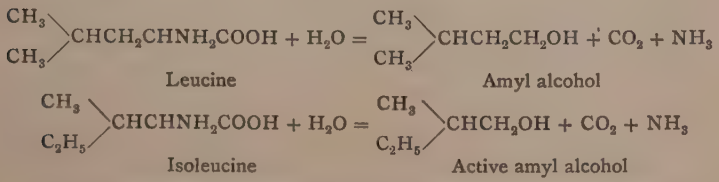
Furthermore, Fischer † has been able to synthesize a diamino acid by the action of ammonia on sorbic acid, an unsaturated acid occurring in the unripe berries of the mountain ash; also another unsaturated acid belonging to the same series as sorbic acid, namely, $\beta$-vinyl acrylic acid, has by the action of ammonia been converted into diamino valeric acid,‡ and further, aspartic acid § has been obtained by the action of ammonia on fumaric acid.

From the plant physiological point of view, however, the interest of these latter discoveries is dependent on the occurrence in the plant both of unsaturated acids and of ammonia.

The researches of Ehrlich || upon the *action of yeast on amino acids* have led to some very interesting results; it was found that the addition of leucine or isoleucine to a fermenting sugar solution gave rise to a production of inactive or

* Erlenmeyer and Kunlin: "Ber. deut. chem. Gesells.," 1902, **35**, 2438.
† Fischer and Schlotterbeck: *id.*, 1904, **37**, 2357.
‡ Fischer and Raske: *id.*, 1905, **38**, 3607.
§ Engel: "Compt. rend.," 1887, **104**, 1805, and 1885, **106**, 1677.
|| Ehrlich: "Ueber die Bedeutung des Eiweisstoffwechsels, etc.," "Sammlung chem. u. chem. tech. Vorträge," Stuttgart, 1911.

active amyl alcohol respectively, according to the following schemes.




The amounts of these alcohols produced are proportional to the quantities of leucine or isoleucine added and rise, under favourable conditions, to as much as 7 per cent; furthermore, it was found that although the leucine parted with its nitrogen in the form of ammonia, the latter substance was not lost, but appeared to be taken up by the yeast in the production of new protein material; this observation led to trying the effect of adding ammonium salts, when it was found that the yeast, finding these latter to be an easier source of nitrogenous food, gave up attacking the leucine, and consequently less amyl alcohol was produced.

These experiments therefore prove that amino acids can be fermented by yeast with the production of alcohols in much the same way as sugars can be fermented. The amino acids of the protein of the yeast cells are the source of the amyl alcohol and succinic acid found among the products of the fermentative activity of such living yeast cells. When yeast juice is employed, these by-products are not formed. The practical importance of these discoveries can be gauged from the fact that the production of amyl alcohol or fusel oil by the yeast fermentation of sugar has always been a source of trouble to spirit distillers, necessitating elaborate processes for refining; these researches have provided both an explanation of the cause and a remedy for the evil.*

Since, moreover, other amino acids besides the leucines are also found to be attacked in a similar way with the production of a number of widely different products, some of which are

* A knowledge of the cause of the amyl alcohol production is also important from the point of view of increasing the yield of this substance, since large quantities of amyl alcohol are required for the preparation of amyl acetate, used as a flavouring material for confectionery, and as a solvent in the manufacture of varnish, smokeless powder, etc.

aromatic, it is easy to account for the different flavours which are peculiar to the various alcoholic beverages, all of which are ultimately produced by alcoholic fermentation of sugars in presence of different proteins.

The destruction of amino acids by enzymes derived from yeasts, fungi or bacteria with the formation of different bye-products, may also account for the flavours of different cheeses, as well as the odour of flowers; the substance phenyl ethyl alcohol, for example, which was produced by the fermentation of phenyl alanine,

$$\underset{\text{Phenyl alanine}}{C_6H_5CH_2CHNH_2COOH} + H_2O = \underset{\text{Phenyl ethyl alcohol}}{C_6H_5CH_2CH_2OH} + CO_2 + NH_3$$

being the chief odoriferous constituent of rose oil.

These researches would therefore lead to the conclusion that the proteins, through the breaking up of various amino acids derived from them, are ultimately responsible for the production of a variety of nitrogen-free alcohols, aldehydes and acids as bye-products, which go to produce the different essential oils, etc.

The metabolism of proteins in the animal world is, as is well known, a very important process and results in their very complete decomposition with the formation of urea, carbon dioxide and water. Although little is known concerning the metabolism of proteins by plants, there is good reason for believing that the destruction of the protein molecule is far less complete; the occurrence of urea has in fact only rarely been recorded in plants, namely, in *Lycoperdon Bovista** and traces have been isolated from *Cichorium endiva*, *Cucurbita maxima*, *Cucumis melo*, *Brassica olereacea*, *B. nigra* and *B. napus*, *Daucus carota*, *Solanum tuberosum* and *Spinacia olereacea*.† It has been suggested that many of the simpler nitrogenous compounds, as, for example, caffeine, theobromine, the alkaloids, skatol and allied substances such as indigo, indoxyl, etc., may be products of protein metabolism.

* Bamberger and Landsiedl: "Monatshefte," 1903, **24**, 218.

† Fosse: "Compt. rend.," 1912, **155**, 851; 1913, **156**, 567, 1938; 1914, **158**, 1374; **159**, 253; Verschaffelt: "Pharmaceut. Weekblad," 1914, **51**, 189.

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# SECTION X.

## ENZYMES.

It has long been known to chemists that the velocity of chemical reactions could, in many cases, be increased by the presence of relatively small quantities of certain substances which do not appear to take any immediate part in the reaction.

This is well illustrated by the familiar example of the effect of a small quantity of manganese dioxide in bringing about the liberation of oxygen from potassium chlorate at a temperature much lower than would be possible by heating this substance alone.

Other examples of the accelerating influence of foreign substances on the velocity of reactions are to be found in the use of cuprous chloride in Deacon's chlorine process, and of spongy platinum, either in the manufacture of sulphuric acid by the contact process, or for effecting the explosive combination of hydrogen and oxygen.

Similarly, the hydrolysis of cane sugar according to the equation—

$$C_{12}H_{22}O_{11} + H_2O = 2C_6H_{12}O_6$$

takes place very slowly in neutral aqueous solutions, but may be greatly accelerated by warming the solution with a little mineral acid.

A feature common to all the above reactions is the fact that the substance which produces the accelerating influence is unaltered by the reaction, and can usually be recovered from the reaction-product unchanged in quality and quantity.

Substances which have this remarkable property of being able in some way to influence the velocity of a reaction, without apparently undergoing any change themselves, and which act in quantities which bear no particular relation to the weights of the reacting substances, are called catalytic agents.

The process of catalysis has been defined by Ostwald as "The acceleration of a chemical change by the presence of some foreign substance," and it must be clearly understood that a catalytic agent only accelerates a reaction, but is not capable of bringing about a reaction which would not take place at all in its absence. Berzelius,* in 1850, drew attention to the similarity between the decomposition of hydrogen peroxide, under the influence of insoluble inorganic catalysts such as platinum or silver, and the decomposition of sugar into alcohol and carbon dioxide under the influence of substances known as ferments. Thus, in view of the ease with which so many complex reactions are effected within the living organism at a low, or a comparatively low, temperature, the idea is suggested that nature likewise makes use of catalysts.

As a matter of fact a large number of complex organic substances, capable of exerting catalytic action, have been isolated from plants and animals; and to these substances the name of enzymes has been applied.

The food of plants, carbohydrate, protein, fats, etc., is, in many cases, valueless unless it can be brought into a condition suitable for assimilation and, very often, translocation. Thus the starch in a leaf must be rendered soluble before it can be transported to other parts of the plants, and, similarly, the starch in a potato before it can be used for the nutrition of the young shoots.

In the living organism these changes are brought about by the enzymes, and, in a word, enzyme action is the strategic centre of vital activity.

With regard to the mode of the formation of enzymes nothing is known; they are generally described as being due to the activity of the protoplasm, a phrase which contains no information. Sometimes the enzymes are secreted in specialized organs or in tissues more or less remote from the cells containing the material to be acted upon. In other cases they are formed in the same cells as the substrate.

A few examples may be given. In *Zea Mais* the cells of the surface of the scutellum next the endosperm have a distinct gland-like appearance, and here and there they dip down into the deeper layers of the scutellum, giving an appearance

* Berzelius: "Jahresber.," 1850, 15, 237, 240, 278.

not unlike the crypts of Lieberkühn of the intestine. These secretory glands of the maize, however, have no lumina. In *Phœnix dactylifera* the secretory organ of the seed is the rounded structure situated opposite the furrow. In *Nepenthes* and other insectivorous plants special glands occur in appropriate places, e.g. in the lining membrane of the pitchers, or in special tentacles, as in *Drosera*.

The fruits of *Rhamnus infectorius* are much used for dyeing. The pericarp contains a glucoside, xanthorhamnetin, which, on hydrolysis, breaks up into glucose and rhamnetin, a yellow compound. This hydrolysis is brought about in nature by an enzyme which is contained in the parenchyma of the raphe o the seed. To illustrate this, the following experiment may be tried.

An aqueous extract of the separated pericarp is made and placed in a glass vessel, then into the solution is thrown the raphe of a seed. A golden yellow precipitate comes down.*

In other cases the enzyme and substrate are contained in different cells of the same tissue, so that it is only necessary to crush up the tissue, or to macerate it, in order to obtain the reaction; the bitter almond, containing emulsin and amygdalin, may be given as an example.

The enzyme-secreting cells of *Zea* and *Phœnix* have been studied by Reed.† He finds that in the resting condition these elements are crowded with granules of a protein naturel which disappear as secretion begins. At the beginning of secretion, the nucleus is poor in chromatin, but this materia, increases in amount as germination proceeds, the nucleolus becoming smaller and smaller. Finally, at the end of the secretory activity, the protoplasm of the gland-cells breaks down, and the products of its disintegration disappear from view.

It may be remarked that in the dried condition enzymes may retain their characteristic power for a considerable time; thus White‡ found that the ferments—diastase, protease and ereptase—of the resting fruits of wheat and barley retained

* Ward and Dunlop: "Ann. Bot.," 1887, **1**, 1.

† Reed: *id.*, 1904, **18**, 267; see also Huie: "Q.J.M.S.," 1897, **39**, 387; 1899, **42**, 203.

‡ White: "Proc. Roy. Soc., Lond.," B., 1909, **81**, 417.

their activity after twenty years, by which time the power of germination is lost. Also, that the subjection of the dry grains to certain extremes of temperature did not destroy the enzymes. Thus the heating of dry oats to 100° C. for four and a half hours was without effect in the destruction of the enzymes; an exposure to a temperature of 130° for one hour, however, did destroy the ferments. On the other hand, a temperature of −200° C. did not destroy the dry diastase of barley

The number of enzymes which a plant may contain is surprising; thus in *Beta vulgaris*, the leaves contain invertase, diastase, and maltase, the stem possesses invertase, diastase, inulase and emulsin, and the root diastase, maltase, inulase, and emulsin, but not invertase.*

The moulds—the digestive activities of which are, to a great extent, extra-cellular—also exhibit marked powers of secreting different enzymes. Thus *Monilia sitophila* may form maltoglucase, trehalase, raffinase, invertase, cytase, diastase, lipase, tyrosinase, and trypsin. These, according to Went,† are secreted according to the nature of the food; Dox,‡ however, who has demonstrated the presence in moulds of protease, nuclease, amidase, lipase, emulsin, amylase, inulase, raffinase, sucrase, maltase, lactase, histozyme, catalase and phytase, considers, from the data at hand, that these enzymes are formed regardless of the chemical nature of the substrate.

Observations such as these open up many questions relating to the nature of enzymes; are all these different ferments really specific, or are there only a few enzyme-nuclei which, before they can attack any particular substance, have to have attached to them certain molecular complexes according to the nature of the substrate?

There may, in certain cases, be made out a curious association of different enzymes. Thus Vines§ found that when a tissue gave the guaiacum reaction, with or without the addition of peroxide, that same tissue also exhibited proteolytic activity and vice versa. Thus in the fruit of the orange, neither the juice nor the pulp gives the guaiacum reaction, whilst, on the

* Robertson, Irvine, and Dobson: "Biochem. Journ.," 1909, 4. 258.

† Went: "Jahrb. Wiss. Bot.," 1901, **36**, 611; see also Pringsheim and Zempter: "Zeit. physiol. Chem.," 1909, **62**, 367.

‡ Dox: "Plant World," 1912, **15**, 40.

§ Vines: "Ann. Bot.," 1903, **17**, 257.

other hand, the peel does. The peel is actively proteolytic, but not the pulp and juice. Similarly the latex of the fig, papaw, lettuce, and spurge, has proteolytic qualities and also gives the peroxidase reaction. The meaning of this association is not clear.

## CLASSIFICATION OF ENZYMES.

The following classification of enzymes, based on the chemical reactions in which they exert their accelerating influence, indicates the extensive use made by nature of these catalysts :—

1. HYDROLYTIC ENZYMES.
   - (*a*) Ester or fat-splitting enzymes (esterases) : Lipase.
   - (*b*) Carbohydrate-splitting enzymes (carbohydrases) :—
     - Invertase which hydrolyses cane sugar to dextrose and levulose.
     - ,, ,, ,, raffinose to levulose and melibiose.
     - Maltase ,, ,, maltose (malt-sugar) to dextrose.
     - Lactase ,, ,, lactose (milk-sugar) to dextrose and galactose.
     - Amylase or Diastase which hydrolyses starch to maltose and dextrin.
     - Inulase which hydrolyses inulin to levulose.
     - Pectinase* which hydrolyses pectose to arabinose.
     - Cytase which hydrolyses hemicellulose to mannose and galactose.
   - (*c*) Glucoside-splitting enzymes :—
     - Emulsin which hydrolyses amygdalin to glucose, hydrocyanic acid and benzaldehyde.
     - ,, ,, ,, β-methylglucoside to glucose and methyl alcohol.
     - Myrosin ,, ,, potassium myronate to allylisothiocyanate, glucose and potassium hydrogen sulphate.
     - Phytase ,, ,, phytin to inósite and phosphoric acid.
   - (*d*) Protein-splitting enzymes :—
     - Pepsin contained in the stomach which hydrolyses proteins to albumoses and peptones.
     - Trypsin ,, ,, pancreas which hydrolyses proteins to polypeptides and amino-acids.
     - Erepsin ,, ,, intestine which hydrolyses proteins to polypeptides and amino-acids.
     - Bromelin ,, ,, pine-apple juice which hydrolyses proteins to polypeptides and amino-acids.
     - Papaïn , ,, juice of the fruit and leaves of the papaw tree (*Carica papaya*) which hydrolyses proteins to polypeptides and amino-acids.
   - (*e*) Urea-splitting enzymes (ureases) :—
     - Ureases obtained from *Micrococcus ureæ* and also from the Soja bean and other seeds† which hydrolyse urea into ammonia and carbon dioxide.

* See p. 146.

† Annett : "Biochem. Journ.," 1914, 8, 449.

2. Fermenting Enzymes.
   (*a*) Alcoholic fermentation of glucose, levulose, mannose, etc., by zymase.
   (*b*) Lactic acid fermentation of lactose by lactic acid bacteria.
   (*c*) Butyric acid fermentation of lactose by the butyric bacteria, etc.
3. Coagulating Enzymes.
   Rennin (Chymosin) which curdles milk.
   Thrombin which coagulates blood.
   Pectase „ „ soluble pectic bodies.
4. Oxidizing Enzymes.
   (*a*) Oxidases which oxidize alcohols to acids, e.g., the action of *Mycoderma aceti*, etc., etc.
   (*b*) Catalases or peroxidases which set free oxygen from hydrogen peroxide, or other peroxides, causing these substances to blue guaiacum resin.

## METHODS EMPLOYED IN ISOLATION OF ENZYMES.

The material from which the enzyme is to be extracted is ground up with water or dilute alcohol together with a little toluene to act as an antiseptic; sometimes the material to be extracted is previously dried by gently warming or by dipping in absolute alcohol; in some cases it is necessary to destroy the cell walls, before extraction, by grinding up with glass or Kieselguhr (infusorial earth) or by autolysis.

From aqueous solutions enzymes may be precipitated in the form of amorphous powders by the addition of an excess of alcohol.

Details for the isolation of certain enzymes are given below.

## CHEMICAL CONSTITUTION.

The chemical constitution and nature of enzymes is, as yet, largely a matter of speculation, owing to the fact that it is very difficult to obtain an enzyme in a pure condition; attempts at purification generally end in the diminution or complete destruction of the activity of the material under examination. Owing to their tendency to be withdrawn (adsorbed) from solution by precipitates formed in their presence, it is difficult to purify them from proteins by any means which involve the precipitation of the latter; this may, to some extent, account for the fact that all enzymes were formerly supposed to be of a protein nature. According to Pekelharing * pepsin is in some way related to the nucleo-

* Pekelharing: "Zeit. physiol. Chem.," 1885, 9, 577.

proteins although it contains no phosphorus; on the other hand, the purest forms of invertase hitherto obtained contain but little nitrogen and do not give the biuret reaction; they are rich, however, in carbohydrate and contain organically combined phosphorus.

Considerable difference of opinion exists in regard to the special class of enzyme known as oxidases. These, according to some authors, as for example Dony-Henault,* are not organic compounds at all, but owe their action to the presence of certain inorganic salts, more especially manganese salts, in colloidal solution. Bertrand,† on the other hand, considers that the laccase of *Rhus succedanea* is a protein, whilst Euler and Bolin ‡ are of the opinion that the laccase of *Medicago sativa* is composed of the calcium salts of glycollic, citric and malic acids.

According to Wolff,§ moreover, a very dilute ferrocyanide solution mixed with a colloidal iron solution gives all the reactions of an oxidase and is partly destroyed by boiling or mixture with traces of metallic salts.

### PROPERTIES OF ENZYMES.

A peculiar property of enzymes, in which they differ from inorganic catalysts, is their sensitiveness to heat and light.

All enzymes are destroyed at 100° and most of them cannot, with safety, be heated much above 60°. The statement made by van't Hoff that the velocity of a reaction is doubled for every 10° rise of temperature is found to hold good for enzymes also with this reservation, that as the temperature approaches a certain height it begins to have a deleterious effect upon the enzyme; the so-called optimum temperature for the activity of any particular enzyme is therefore a compromise between the maximum acceleration effect, which can be attained in accordance with van't Hoff's rule, and the maximum temperature to which the enzyme can be heated without undergoing destruction. Inasmuch as the enzymes themselves are not living—unless, indeed, we consider the phenomena of life to be due to the activities of a complex of

* Dony-Henault: "Bull. Acad. Roy. Belg.," 1908, 105.
† Bertrand: "Ann. Chim. Phys.," 1907, [7], **12**.
‡ Euler and Bolin: "Zeit. physiol. chem.," 1909, **61**, 1.
§ Wolff: "Compt. rend.," 1908, **147**, 745.

enzymes—the sensitiveness of an active enzyme, dissociated from the living cell, to heat is most readily explained by attributing it to the colloidal nature of the enzyme with the consequent tendency to coagulation by heat.

With regard to the action of light rays on enzymes it appears, according to Iodlbauer and v. Tappeiner,* that there exist two distinct kinds of action:—

(*a*) Those produced by ordinary light in the presence of oxygen, and (*b*) those produced by ultra-violet light independently of oxygen.

The destructive action which has resulted from exposure to bright sunlight therefore appears to be dependent on the presence of oxygen, and is greatly increased by the presence of fluorescent substances, such as eosin, quinoline red, etc.†

It was first shown by Green‡ that ultra-violet light destroyed diastase, and since then several other authors have described similar effects for other enzymes.§

The action of radium and radium emanation on enzymes has been studied by Wilcock,‖ by Loewenthal and Edelstein,¶ by Bickel, by Loewenthal and Wohlgemut, and others.**

The influence of various chemicals on the activity of enzymes will be dealt with later under the heading of "paralysers".

### COLLOIDAL NATURE OF ENZYMES.

A fairly detailed account of the nature of colloidal solutions has been given above; it will suffice, therefore, merely to mention here that enzymes possess most of the more important properties of such solutions.

Foremost amongst these properties is their want of diffusibility; as already pointed out, this does not mean that they are quite unable to diffuse, but rather that their rate of diffusion is very small.†† Their ability to diffuse through a membrane—commonly known as dialysis—is largely dependent on the nature or structure of the membrane, but, as a

* Iodlbauer and v. Tappeiner: "Deut. Archiv. Klin. Med.," 1906.
† Tappeiner: "Biochem. Zeit.," 1908, **8.**
‡ Green: "Trans. Roy. Soc., Lond.," 1897, **188**, 167.
§ E.g. Burge, Fischer, and Neill: "Amer. Journ. Physiol.," 1916, **40**, 137, 426.
‖ Wilcock: "Journ. Physiol.," 1907, **34.**
¶ Loewenthal and Edelstein: "Bioch. Zeit.," 1908, **14**, 484.
** Loewenthal and Wohlgemut: *id.*, 1909, **21**, 476.
†† Chodjajew: "Arch. Phys.," 1898, 241.

general rule, enzymes will not pass through a parchment membrane; nevertheless Fränkel and Hamburg found that on subjecting a sample of diastase obtained from malt to dialysis, they were able to effect a separation into two distinct enzymes; one of these passed through the parchment and was found to be a sugar-producing enzyme, while the other which would not diffuse was able to liquefy starch.

On the other hand, most enzymes will pass through a porcelain filter, a fact which is made use of for separating the active enzymes from living cells. Owing to adsorption on the surface of the porcelain the filtration may, however, be accompanied by considerable loss of enzyme.

Enzymes for the most part are soluble in water, or in dilute salt solutions, or in glycerin. The lipases or fat-splitting enzymes, however, whether of animal or of vegetable origin, are characterized by their slight solubility in water.

On the other hand, enzymes are precipitated from solution by alcohol and by neutral salts such as ammonium sulphate.

Enzymes exhibit in a marked degree the phenomenon of adsorption,* and consequently are liable to be withdrawn out of solutions by other substances, such as calcium phosphate or uranyl phosphate, which may happen to be precipitated in their presence. For the same reason they are extracted from solution by shaking with charcoal, china clay, etc. The conditions obtaining here are to a large extent dependent on the electric charges of the substances concerned, a question which has been considered in detail by Michaelis.†

### MODE OF ACTION OF ENZYMES.

To explain the mode of action of inorganic catalysts, it is frequently supposed that they form labile additive compounds with one of the reacting substances which then react more readily than the original substance would have done.

Similarly, in the case of the enzymes, it is now generally assumed that they enter into some form of loose combination with the substrate; in spite of this the enzyme is, in general, not altered by the reaction but retains its original activity

* See Dauwe: "Hofm. Beitr.," 1905, **6**, 426.

† Michaelis: "Biochem. Zeit.," 1908, **10**, 283; "Dynamik d. Oberflächenwirkung," Leipzig, 1909.

after having completed its work, unless, of course, the products of the reaction have any effect on it.

In the group of carbohydrates the action of the enzymes is usually regarded as being more or less specific, each disaccharide being hydrolysed only by its own enzyme, e.g. cane sugar by invertase, milk sugar by lactase, and malt sugar by maltase.

That this specific activity is in some way connected with the molecular structure of the substances would appear from the researches of Fischer on the action of enzymes upon artificial glucosides. Fischer, by the action of methyl alcohol and hydrochloric acid on glucose, obtained two stereoisomeric methyl glucosides known respectively as the $\alpha$ and $\beta$ variety. Now these two substances differ from each other only by the arrangement in space of the groups attached to the terminal carbon atom, and it is found that while the $\alpha$ modification is readily converted by maltase into glucose and methyl alcohol, the $\beta$ modification is not affected by maltase at all, but is, on the other hand, hydrolysed by emulsin, which has no action on the $\alpha$ compound.

It would appear from this that the structure of the molecule which is to be decomposed is the determining factor.

Incidentally it may be mentioned that the fact that emulsin and maltase are complementary in their action upon $\alpha$ and $\beta$ methyl glucosides, enables one to classify a glucoside as belonging to the $\alpha$ type if it is attacked by maltase and not by emulsin, or to the $\beta$ type if it is attacked by emulsin and not by maltase.

Several other examples of this selective action on the part of enzymes for different optical isomers have been described by Fischer and Abderhalden, who found that whereas d-alanyl-d-alanine, d-alanyl-l-leucine were split up by enzymes, their stereoisomers d-alanyl-l-alanine and l-alanyl-d-alanine were not.

This peculiar dependence upon structure has led Fischer to suggest that the relationship which exists between the substance to be decomposed and its enzyme is similar to that existing between a lock and its key ; or, in other words, unless the molecular structures of the two substances fit each other no interaction can take place.

These facts, of course, give strong support to the theory of

he formation of some sort of compound between the enzyme and the substrate.

It should, however, be noted that the action of enzymes is not entirely specific, inasmuch as the one and the same enzyme may be able to hydrolyse two or more substances. Thus maltase is able to hydrolyse both maltose and $\alpha$ methyl glucoside; and emulsin is able to decompose $\beta$ methyl glucoside, $\beta$ methyl galactoside, milk sugar, amygdalin (the glucoside of bitter almonds, and with which it is primarily associated in nature), arbutin, salicin, and coniferin.

The specific nature of the interaction between enzymes and other substances is, however, only really strongly marked in connexion with optically active substances. For, taking the case of the fat-splitting enzymes or lipases, practically all esters are broken up by pancreatic lipase, although the ease with which the hydrolysis is effected may vary considerably in different cases.

On the other hand, Fischer and Abderhalden have shown that whereas pancreas extract was able to hydrolyse a number of artificial polypeptides, it was quite unable to act upon others.

Fischer has described enzymes as optically active catalysers, and explains in this way how it is that they produce optically active substances from inactive material, as, for example, when moulds, such as *Penicillium glaucum*, *Aspergillus niger*, etc., are allowed to grow upon inactive tartaric acid with the formation of l-tartaric acid; it is assumed that the enzymes combine with the racemic substrate to form isomeric substances which decompose at different rates and so form optically active products.

A most important contribution to the elucidation of the activity of enzymes is the discovery of the stimulating or inhibiting influence exercised by certain substances which may be described respectively as Activators and Paralysers.

### ACTIVATORS.

Under this heading are included substances described by different authors as Co-enzymes * or Accelerators. In some

* Bayliss distinguishes between co-enzymes and accelerators by reserving the former term for those substances without which the enzyme is unable to

cases the substances are quite simple chemical individuals, such as acids, alkalis, or salts, and in others they may be complex and of unknown constitution, as in the case of the co-enzyme of zymase (but see p. 379). They have, however, properties in common, namely that they can be separated from the enzymes by dialysis, and are not destroyed by heating. Moreover, an enzyme rendered inactive by removal of its co-enzyme can be restored to its original activity by mixing again with this substance.

This latter effect has been shown especially for dialysed liver extract which has no lipolytic action; if, however, this extract be mixed with some of the solution which had been dialysed out, or with boiled liver extract, the characteristic lipolytic action is regained. In this case it can be shown that the bile salts are the active co-enzyme. Similarly, the activity of zymase, the enzyme of yeast cells, is dependent on the presence of certain complex phosphoric esters, which, likewise, can be separated from zymase by dialysis and are not destroyed by boiling water.

Other examples of the dependence of the enzyme upon activators are the necessity for the presence of a small quantity of acid in order that pepsin and the lipase of castor-oil seeds, for instance, may exert their respective actions.

Similarly trypsin requires a faintly alkaline medium to exert its proteoclastic action; in many cases the presence of calcium salts is essential, as, for example, in the clotting of milk by rennin, the clotting of blood by thrombin, and the gelatinization of pectin by pectase.

In the case of some enzymes the substance at its seat of origin is not a true enzyme but a so-called proferment or zymogen which is not itself active but only becomes so on being brought into contact with another more or less complex substance known as a kinase. Thus, for example, trypsinogen, which is contained in pancreatic juice, has very little action on proteins but is converted into the true proteolytic enzyme trypsin on coming in contact with the kinase—entero-kinase

exert its activity, and the latter for substances which stimulate or accelerate a reaction without being absolutely essential to its taking place; an example of the latter class is furnished by traces of manganese salts which greatly increase the oxidizing power of the enzyme laccase, though it has yet to be proved that the laccase is unable to act in their absence.

—which is secreted by the mucous membrane of the duodenum.

The relation between proferment and kinase is different from that existing between enzyme and co-enzyme inasmuch as the two latter can be alternately mixed and separated; on the other hand, the reaction between proferment and kinase is not reversible; furthermore, the proferment is not really regarded as a complete ferment, while the true ferment produced from it, by combination with the kinase, may still be dependent upon an activator for its activity.

The following example of the dependence of thrombin upon calcium salts will illustrate this; the coagulation of blood by thrombin consists in the conversion of the soluble substance fibrinogen into the insoluble substance fibrin. The blood plasma contains a proferment thrombogen and also calcium salts, but these two substances alone are unable to coagulate fibrinogen. When, however, the blood is drawn, a kinase, known as thrombokinase, which is secreted by the blood corpuscles, combines with the proferment thrombogen forming the true coagulating ferment thrombase.

Both enterokinase and thrombokinase are destroyed by heat.

### PARALYSERS.

Paralysers are substances which reduce or destroy the activity of enzymes. These may be either the products of the activity of the enzyme or of foreign substances. Examples of the first class are the acetic or lactic acids which, unless neutralized, destroy the ferments producing them; similarly the alcohol produced by the fermentation of sugar ultimately stops the fermentation. Also, Tammann has shown that the hydrolysis of amygdalin by emulsin was retarded by the addition of any one of the products of hydrolysis, namely glucose, benzaldehyde or hydrocyanic acid, but most markedly by the latter. Similarly Croft-Hill found that glucose interfered with the action of maltase, and the Armstrongs * likewise have pointed out a number of examples of the inhibiting action of the reaction-products upon the enzyme.

Amongst foreign substances having a retarding effect on

* Armstrong: "Proc. Roy. Soc., Lond.," B., 1907, 79, 360.

enzymes may be mentioned inorganic substances such as mercuric chloride or cyanide, arsenious oxide, sulphuretted hydrogen, ozone, and organic compounds such as chloroform, chloral, formaldehyde, hydrocyanic acid, phenylhydrazine aniline, alcohol, etc.; the influence of these substances on different enzymes varies considerably; thus, for example, alcohol usually acts as a paralyser, but on lipase it has a stimulating effect.

The majority of the substances included in the above list also act as poisons to colloidal solutions of metals; the peculiar phenomenon of the recovery of metallic colloidal solutions from poisoning by hydrocyanic acid, is also met with in the case of the enzymes, and is likewise attributed to the oxidation of the poison.

The mechanism of toxic action is as yet unexplained; it is assumed that some form of chemical combination between the paralyser and the substrate enzyme or activator takes place.*

The work of Caldwell † on the effect of toxic agents upon bromelin, the proteolytic enzyme of the pineapple fruit, may be cited in illustration. The prepared enzyme (see p. 373) was dissolved in water so that each cubic centimeter contained ·006 gram and the solution was rendered acid or alkaline by the addition of hydrochloric acid or sodium hydrate of a concentration of M/32. Five c.c. of the enzyme solution was placed in a series of test tubes together with 1 gram of boiled granulated egg albumen; then to each tube was added a solution of the paralyser.

The tubes were then placed in a water bath and kept at a temperature of 40° C. for twenty-four hours. At the end of this period, the liquids were filtered, if necessary, to remove any albumen, and tested for peptones, leucine and tyrosine by the biuret and tryptophane reactions, confirmatory tests being applied if necessary.

In the following table the metals are arranged in their order of toxicity, the top ones being the most poisonous, and are compared with the results obtained by Matthews ‡

* Cf. Loewenhart and Kastle: "Amer. Journ. Chem.," 1903, **29**, 397, 563.
† Caldwell: "Bot. Gaz.," 1905, **39**, 409.
‡ Matthews: "Ann. Journ. Physiol.," 1904, **10**, 290; 1904, **11**, 455.

on the eggs of *Fundulus heteroclitus* and McGuigan* with diastase.

| Matthews. | McGuigan. | Caldwell. |
|---|---|---|
| Ag | Ag | Ag |
| Hg | Hg | Hg |
| Cu | Cu | Cu |
| Cd | Cd | Pb |
| Pb | Co | Zn |
| Zn | Zn | Ba |
| Co | Pb | Cd |
| Li | Sr | Co |
| Sr | Ba | Na |
| Na | Mg | Li |
| Ba | Li | Sr |
| Mg | Na | Mg |
| $NH_4$ | | $NH_4$ |
| K | | K |

With regard to salts, Caldwell found that nitrates inhibit the action of the enzyme in somewhat greater dilution than the corresponding sulphates and chlorides. He agrees with Matthews that the affinity of the atom or ion for its electrical charge is the main factor which determines its physiological action.

It is to be remembered that the effect of poisons vary with the purity of the preparations used; a slight admixture of proteins and other impurities makes it necessary to increase the concentration of the poison greatly in order to inhibit the enzyme action.

### ANTI-ENZYMES.

The term anti-enzyme is applied to a class of substances occurring in the living organism or produced in it by subcutaneous injection with an enzyme. The anti-enzymes are antagonistic in their action upon the enzymes, and their action is quite specific, the relationship between an enzyme and its anti-body being similar to that existing between a toxin and an anti-toxin. The first example of immunity against an enzyme was recorded by Hildebrandt,† the enzyme being emulsin.

* McGuigan: *id.*, 1904, 10, 444.
† Hildebrandt: "Virch. Arch.," 1893, 131, 12, 26.

Since then, anti-enzymes have been discovered for lipase, amylase, pepsin, papaïn and urease. Anti-trypsin and anti-rennet occur normally in the blood, and, according to Weinland,* anti-pepsin and anti-trypsin occur in the mucous membranes of the stomach and intestine respectively.

In this connexion the work of Czapek † on the anti-ferment reaction in tropistic movements of plants is of particular interest. It is impossible here to give a complete account of the investigations referred to, but the following facts will give some idea of the phenomena under discussion.

1. It was found that the roots of *Vicia Faba* when subjected to the stimulus of gravity always reduced silver more effectively than unstimulated roots. The mode of testing is as follows. The roots, stimulated or otherwise, are cut into longitudinal slices and boiled in an ammoniacal solution of silver nitrate; the darkness assumed by the preparations is an indication of the amount of silver reduced.

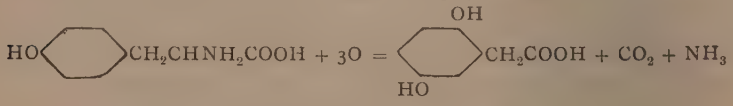
2. If the roots of the lupin, for example, be anæsthetized, a deposition of tyrosine, in the shape of spherical crystals, takes place; but, strangely enough, not in the root-tip, nor in the youngest parts of the growing regions where the maximum reduction of silver takes place.

The question naturally arises, is there any connexion between the reduction of silver and the production of tyrosine? As a matter of fact there is, for it was observed that in roots containing much crystalline tyrosine, although at first the tyrosine-containing cells did not reduce silver, they did so eventually, the silver reducing properties becoming more and more marked as the tyrosine disappeared, so that eventually a very strong reduction obtained.

By these and similar observations it was shown that the tyrosine disappears by means of a ferment, tyrosinase, and that one of its products is the substance which reduces the silver. Tyrosinase appears to be widely distributed in plants, e.g. it has been identified in *Russula*, in the tubers of the *Dahlia*, in the beetroot, as well as in root-tips. The product of the decomposition of tyrosine referred to above is homo-

* Weinland: "Zeit. f. Biol.," 1903, 44, 45.
† Czapek: "Ann. Bot.," 1905, 19, 75.

gentisic acid ($C_8H_8O_4$),* a substance which is also produced in a similar fashion in the human body.†




Tyrosine Homogentisic acid

Homogentisic acid may be prepared from root-tips by grinding them in 96 per cent alcohol, filtering off the solids, and evaporating over a water bath. The residue when dissolved in water forms a brown solution which is free from sugar, and gives a faintly acid reaction. In the air it turns dark.

The following are characteristic reactions.

1. Treated with alkali it turns reddish-brown.
2. It reduces an ammoniacal solution of silver nitrate on warming.
3. Fehling's solution is feebly reduced by it on warming.
4. Ferric chloride gives a green coloration.
5. Ferric sulphate gives a violet-blue coloration.
6. Millon's reagent gives a yellow colour.
7. Homogentisic acid is precipitated by lead acetate.
8. It also gives a reddish colour with hydrogen peroxide.

Czapek further found that if ground-up root-tips were kept for some time under the influence of chloroform, the mass gradually loses its power of reducing silver, and even small quantities of homogentisic acid intentionally added to the preparation gradually disappear; this is due to the action of an oxidase.

It has been seen that root-tips stimulated by gravity give a strong reduction of silver, but not so the unstimulated roots. To explain this there are two alternative hypotheses; either the action of the oxidase is inhibited during geotropic stimulus, so that the homogentisinic acid, which is otherwise acted upon, disappears more slowly than under ordinary circumstances and so accumulates, or, there is a diminution in the production of oxidase by the root-tip.

* The occurrence of this acid in plants is denied by Schultze and Castoro: "Z. physiol. Chem.," 1906, **48**, 387, 396. See also Bertel: "Ber. deut. bot. Gesells.," 1902, **20**, 454.

† Wolkow and Baumann: *id.*, 1891, **15**, 260; Huppert: "Z. physiol. Chem.," 1897, **23**, 412; Garrod and Hele: *id.*, 1905, **33**, 198.

From experimental evidence, Czapek considers that the first alternative is the true one. He found reason to believe that the inhibiting substance is an anti-oxidase of some potency which is precipitated by alcohol, destroyed by heat (62°) and may be isolated by filtration through a porcelain candle. Czapek further ascertained that the anti-oxidase is more or less specific, for although the oxidase and the anti-oxidase of closely related plants have a mutual action, this is not so for plants widely separated.

It is supposed that the anti-oxidase is only produced as a result of gravitation stimulus, so that the simple reaction is as follows. The tyrosine is converted by the action of tyrosinase into homogentisinic acid; the further oxidation of the acid by oxidase is inhibited by the production of an anti-oxidase which renders the oxidase more or less inefficient, and so the homogentisinic acid accumulates.

For the mode of quantitatively determining the amount of homogentisinic acid and for other details, Czapek's paper must be consulted.

The interaction between enzyme and antienzyme appears to be of the nature of adsorption.

### ENZYMES AND THE LAWS OF MASS ACTION.

According to the Law of Mass Action enunciated by Guldberg and Waage, the rate at which a body undergoes chemical change is dependent on the concentration as measured by the number of gram molecules of substance present in the litre; consequently the amount of substance changed in unit time will be greater at the beginning of the reaction than towards the end, since the amount of unchanged substance is continually decreasing.

The relationship between the amount of substance $x$ (measured in gram molecules per litre) changed in time $t$ (measured in minutes) and the original concentration $a$ of the substance is given by the equation:—

$$K = \frac{1}{t} \log \frac{a}{(a-x)}$$

The above formula holds only for the decomposition of a single substance, and it is, therefore, characteristic of what is known as a Monomolecular reaction or a reaction of the first

order, and as such is applicable to all cases of hydrolysis, as for example:—

$$C_{12}H_{22}O_{11} + H_2O = 2C_6H_{12}O_6$$

Although from the left-hand side of the equation it would appear that two substances are reacting, the quantity of water present is so large, as compared with the amount of cane sugar, that its concentration is practically unaltered, and therefore, for all intents and purposes, only a single substance is undergoing alteration in concentration.

Now the hydrolysis of cane sugar which takes place slowly in aqueous solution is catalytically accelerated by the addition of dilute mineral acids, the effect being greater in proportion to the amount of acid used, without, however, altering the order of the reaction; in just the same way enzymes accelerate hydrolyses in accordance with the law of mass action for monomolecular reactions, thereby showing that they are true catalysts.

In reactions acting in accordance with the logarithmic equation above given, the amount of substance changed in a given time bears a constant ratio to, or is a constant fraction of, the amount of substance unchanged; on plotting the amounts changed as ordinates against the time as abscissæ there is accordingly obtained what is known as a logarithmic curve.

Now it is found that when this is done for an enzyme reaction the curve both at the beginning and at the end of a reaction is not logarithmic but linear. Thus Horace Brown and Glendinning * found that equal amounts of starch were hydrolysed by diastase in equal times during the earlier part of the reaction, in other words, the course of the reaction was expressed by a straight line; as the reaction proceeded, however, it became logarithmic, or, in other words, at the commencement, when the concentration of the substance being hydrolysed is great as compared with that of the enzyme, the reaction is linear and not in accordance with the law of mass action, but where the concentration of the enzyme is great as compared with that of the substance being hydrolysed, the reaction obeys the law of mass action.

* Brown and Glendinning: "J. Chem. Soc., Lond.," 1902, **81**, 392.

Similar results were obtained by Adrian Brown* in the study of the action of invertase on cane sugar; he also expresses the view that, in the case of alcoholic fermentation and other enzyme actions which do not apparently conform with the law of mass action, the exceptional action "is due to a time factor accompanying molecular combination and change which limits the influence of mass action . . . this theory demands not only the formation of a molecular compound of enzyme and reacting substance, but the existence of this molecular compound for an interval of time previous to final disruption and change".

Similarly E. F. Armstrong† in studying the action of lactase and maltase upon their respective sugars found that while the reaction was in the main logarithmic, both the initial and final stages were linear; this is explained by the fact that as a result of the combination between the enzyme and the substrate there will be an excess of substrate at the commencement but an excess of enzyme at the end, both of which conditions favour a linear change.

On calculating the velocity constant for that part of the reaction which is logarithmic it is found that, as a rule, the value steadily decreases, or, in other words, the enzyme appears to become less active. This may be accounted for in one of two ways: either by the assumption that the products of the reaction combine with the enzyme or, by their concentration, exercise some inhibiting influence upon the enzyme; or else by assuming that the tendency for the reverse action to take place has a retarding effect.

That there should be a tendency for the reverse reaction to take place is a perfectly legitimate conclusion; in fact van't Hoff long ago pointed out that a catalyst which accelerates a reaction in one direction must also be able to exert an accelerating effect on the reverse reaction. Consequently the same enzymes which effect hydrolyses should also, under suitable conditions, be able to synthesize.

The first experimental proof of this was given by Croft Hill,‡ who showed that when maltase was allowed to act on

* Adrian Brown: "J. Chem. Soc., Lond.," 1902, **81**, 379.
† Armstrong: "Proc. Roy. Soc., Lond.," B., 1904, **73**, 500, 516, 526; **74**, 188, 195.
‡ Croft Hill: "J. Chem. Soc. Lond.," 1898, **73**, 634.

a concentrated solution of glucose, the disaccharide iso-maltose was produced; later it was shown * that the disaccharide iso-lactose could be synthesized from galactose and glucose by the action of lactase from Kefir. Since then a large number of enzymatic syntheses have been effected, amongst them being included the synthesis of maltose itself by the action of emulsin on a concentrated solution of glucose which was described by E. F. Armstrong, and also the formation of glycogen from a 30 per cent solution of fructose by yeast-extract free from glycogen,† a reaction which most probably involves the conversion of fructose into glucose.

The following experiment has been devised by Bayliss‡ for demonstrating the synthetic action of emulsin in the production of a glucoside:—

Two solutions are required: (*a*) a 15 per cent solution of hydroquinone in glycerol, and (*b*) a 50 per cent aqueous solution of glucose mixed with an equal volume of glycerol. The two solutions (*a*) and (*b*) are mixed in equal proportions, and about 2 per cent of emulsin are added, and the mixture is ground in a mortar and then warmed to 38° to make it less viscid. Ten per cent of the solution are then delivered by means of a pipette into a series of test tubes, a little toluene is added to each, and after displacing the air above the liquid by $CO_2$, the tubes are sealed up in a blow-pipe, but this is not absolutely necessary; if this is not done the liquid may darken owing to oxidation of the hydroquinone, but on adding a small quantity of sodium bisulphite the colour is discharged.

One sample is at once diluted to 50 c.c., and filtered, and its rotation is measured in the polarimeter. The other tubes are placed horizontally in an incubator, and samples are withdrawn every three or four days; the tubes are cracked across, and their contents are rinsed into a 50 c.c. flask.

The initial rotation of about + 3° will be reduced to 0·5° in a week, indicating a synthesis of 25 to 30 per cent of arbutin.

To prove that the diminution of rotation is not due to destruction of glucose the glucoside may be reconverted into

* Fischer and Armstrong: "Ber. deut. chem. Gesells.," 1902, **35**, 3144.

† Cremer: *id.*, 1899, **32**, 2062. See also Meyer: "Bot. Ztg.," 1899, **57**, 313.

‡ Bayliss: "J. Physiol.," 1912, **43**, Proceedings, xl.

its components by diluting a sample to 50 c.c. and adding half a gram of fresh emulsin. In about two to three days the hydrolysis will be complete, and the original rotation will be restored. As commercial emulsin is, however, itself lævo-rotatory, a control experiment should be made with emulsin alone or else the emulsin may be precipitated by adding mercuric nitrate, and filtering before the polarimeter reading is made.

In a subsequent investigation * Bayliss found that actually very little arbutin was formed, but that the glucoside really produced was that of glycerol. That being so, the hydroquinone can be left out of the mixture, and as a result the sealing of the tubes and subsequent treatment with sodium bisulphite is rendered unnecessary. The best mixture is made as follows: Glucose (anhydrous) 18, water 12, glycerol 40, and emulsin 3 parts by weight. The glucose must be dissolved in the water and cooled before adding the glycerol, owing to the production of glycerol glucoside by heat. At a temperature of 47° a diminution of rotation from + 2°·83 to + 0°·80 takes place in seven days, and equilibrium at − 0°·16 (corresponding to about 75 per cent synthesis) is practically attained in fifteen days.

The experiment is of interest with regard to the theory of catalysts, since if the above mixture of glycerol, hydroquinone and glucose is heated without enzyme to 100° for some hours a certain amount of glucoside synthesis results; it may, therefore, be assumed that some synthesis also takes place, though very much more slowly, at 38°. The emulsin, therefore, in accordance with Ostwald's definition of an enzyme, merely accelerates a reaction which is already taking place, though very slowly.

### A CONSIDERATION OF CERTAIN TYPES OF ENZYMES.

Before passing on to a more special consideration of individual enzymes, attention must be drawn to a point of general application to all enzymes. It is of the highest importance that experiments on enzymes which take several hours to carry out, should be conducted under aseptic conditions in order to avoid bacterial activity. The fermenting mixtures obviously cannot

* Bayliss: "J. Physiol.," 1912, 44, Proceedings, ix, and 1913, 46, 236.

be sterilized by means of heat, so that antiseptics must be added. Amongst those commonly employed are chloroform, toluene, thymol, sodium fluoride, and hydrocyanic acid. The nature of the antiseptic exerts a considerable influence upon the activity of the enzyme used, so that it is necessary to try many different antiseptics. The following table illustrates this in the case of papaïn* :—

| Reaction. | Hydrocyanic acid. | Chloroform. | Sodium fluoride. |
|---|---|---|---|
| Acid (·5 per cent citric acid) | Fibrin quite disintegrated<br>Marked trypto-phane reaction | Scarcely attacked<br>Faint trypto-phane reaction | Distinctly attacked<br>Faint trypto-phane reaction |
| Alkaline (5 per cent $Na_2CO_3$) | Fibrin quite disintegrated<br>Faint trypto-phane reaction | Scarcely attacked<br>Doubtful trypto-phane reaction | Scarcely attacked<br>Doubtful trypto-phane reaction |
| Neutral | Fibrin nearly all gone<br>Distinct trypto-phane reaction | Distinctly attacked<br>Faint trypto-phane reaction | Mostly disintegrated<br>Faint trypto-phane reaction |

## LIPASE.

The existence of a fat-splitting enzyme or lipase in the animal kingdom has long been known. This substance, which is known as steapsin, is contained in the pancreas; acting in an alkaline medium it is able to break up fats into glycerol and free fatty acids, the latter combining in the intestine with alkali to form the sodium salts or soaps. Umeda† finds that phosphates are the most active constituents of the coenzyme of inactive lipase obtained from pancreatic extracts.

In 1890 Green‡ found that germinating seeds containing fat or oil, when macerated with water and left for some time, gradually acquired an acid reaction. This observation was subsequently confirmed and extended by Connstein, Hoyer,

* Vines: "Ann. Bot.," 1903, **17**, 602.
† Umeda: "Biochem. Journ.," 1915, **9**, 38.
‡ Green: "Proc. Roy. Soc., Lond.," 1890, **48**, 375.

and Wartenberg,* with the result that it has been found that the seeds of Euphorbiaceæ, and especially castor-oil seeds, whether germinating or not, contain an enzyme capable of hydrolysing fats. Lipase may occur in the seed as in the castor-oil, or it may develop during germination as in linseed. The fact that hydrolysis is slow at first and then suddenly increases from 5 per cent after one day to 58 per cent after two days and to 95 per cent after four days led Connstein to the conclusion that for rapid hydrolysis a certain minimum amount of free acid must be present, and it was found that when a little free acid was added from the commencement hydrolysis could be completed within a few hours. Similar observations regarding the curve of the hydrolysis of fats during the germination of *Ricinus* seeds have been made by Delcano.†

According to Nicloux‡ fats may be attacked by other means. He used castor-oil seeds, which were ground up and the cytoplasm separated from the aleurone grains and other cell contents by mechanical means.

It was found that the cytoplasm thus prepared showed a marked power of hydrolysing fats, acting in the same way as an enzyme and obeying the laws of enzyme action. But inasmuch as the active substance, which Nicloux calls lipaseïdine, contained in the protoplasm is destroyed by water as soon as its protective layer of fat is removed, it is not considered to be an enzyme in the ordinary sense of the term.

The most favourable conditions for the activity of lipase may be summarized as follows :—

(*a*) The presence of free acid varying from N/60 to N/100 or less, according to the amount of material to be hydrolysed.

(*b*) The presence of a certain amount of water.

(*c*) The formation of a good emulsion.

(*d*) The maintenance of a suitable optimum temperature, varying from about 23° to 42° C.

### THE ISOLATION OF LIPASE.

Lipase may be separated from the seeds of *Ricinus* by the following means : The seeds are allowed to begin germination ;

* Connstein, Hoyer, and Wartenberg : "Ber deut. chem. Gesells.," 1902, **35**, 3988 ; Hoyer : *id.*, 1903, **37**, 1441 ; "Zeit. Physiol. Chem.," 1907, **50**, 414.

† Delcano : "Centrlbl. Bakt.," 1909, **24**, 130.

‡ Nicloux : "Proc. Roy. Soc., Lond.," B., 1906, **77**, 454.

when the radicles have protruded a little way, the endosperms are ground in a mortar with a 5 per cent solution of sodium chloride. The liquid is filtered off and placed in a dialyser; the salt having thus been removed, an excess of alcohol is added and the precipitate filtered off. The precipitate is washed with alcohol and may be dissolved in water before use.

For commercial purposes the enzyme is prepared as follows:* Castor-oil seeds are ground up with water and then centrifuged; the resulting emulsion, which contains castor oil, proteins, and the enzyme, is then allowed to ferment at a temperature of 24°, whereby a scum containing the ferment rises to the surface and can be separated from the aqueous layer. This scum is then allowed to act upon the molten fat in the presence of water and a little manganese sulphate as a catalytic agent.

The following experiments described by Connstein, Hoyer, and Wartenberg, may be taken as an illustration of the process on a small scale.

Five grams of castor-oil seeds are macerated with 10 c.c. of water containing 0·2 gram of acetic acid and 0·1 gram of chloral hydrate. After twenty-four hours it is found that about 58 per cent of the fat originally present has been hydrolysed.

To show that the enzyme is not destroyed by extracting the fat from the seeds by means of ether, 1·5 grams of seeds which had been so extracted were ground up with 75 grams of cotton-seed oil and 15 grams of N/10 sulphuric acid. In forty-four hours 82 per cent of the oil had been hydrolysed.

### QUANTITATIVE DETERMINATION OF THE ACTIVITY OF LIPASE.

The activity of this enzyme is conveniently studied by allowing it to act on ethyl butyrate and observing the amount of acid liberated by titration or by conductivity measurements.

## DIASTASE.

Diastase is one of the commonest of enzymes; in fact it may be regarded as being universally present in the higher plants. The amount present in any particular organ varies according to the conditions obtaining; thus when the temperature and other factors are most favourable for growth and

* Cf. Hoyer: "Der Seifenfabrikant," 1905, 25, No. 27.

for the germination of starchy seeds, diastase is much more abundant than when growth and germination are sluggish. Also, the amount of diastase is always greater in starch leaves than in sugar leaves and the same holds for insolated leaves containing much starch, as compared with shaded leaves containing little or no starch.*

According to Cloizaszcz and Josch,† ordinary diastase consists of two enzymes; one liquefies starch, which is then converted into sugar by the activity of the other.

### ISOLATION OF DIASTASE.

To obtain a relatively large quantity of diastase, germinated barley gives excellent results. The grains are soaked in water for twelve hours, and then spread out in a thin layer on a tray which is placed in a warm, damp—but not too damp—place. When the radicles are about one quarter of an inch long, the grains may be dried at a temperature not exceeding 40° C.; they are then ground up as finely as possible. The powder is mixed thoroughly with about four times as much water, and allowed to stand for an hour or two, the mixture being well shaken up periodically. The fluid is then filtered off and evaporated in a vacuum to a small bulk; this concentrated solution is poured into an excess of absolute alcohol, whereby the diastase, and other substances, are precipitated. The precipitate is filtered off, and washed with alcohol. The diastase thus obtained may be partly purified by dissolving in water and re-precipitating with alcohol.

Although diastase occurs in green leaves, it is often difficult to demonstrate its presence in an aqueous extract of the fresh tissue. If the leaves be dried and ground to a very fine powder, the above procedure should yield positive results; if not, then the powdered leaves may be added directly to a one per cent solution of starch paste or to a little dry starch suspended in water in a watch glass. The disappearance of the starch, as indicated by the iodine reaction, and the corrosion of the solid starch grains, point to the presence of diastase.

* Eisenberg: "Flora," 1907, 97, 347.
† Cloizaszcz and Josch: "Biochem. Zeitsch.," 1917, 80, 211.

The action of this enzyme is promoted by the presence of acids, e.g. hydrochloric or citric, but if too much acid be added, the action is inhibited.

To study the action of diastase on starch a mixture of these two substances may be tested from time to time with iodine solution.

Appleman * gives the following experiment. A number of test tubes, say ten, each containing 1 c.c. of a one per cent solution of starch paste, are placed in ice. The extract of the material to be examined for its diastatic activity is added to the mixture in increasing amounts. Thus to the first tube is added 1 c.c. of extract, to the second 1·1 c.c., to the third 1·2 c.c., and so on. A drop or two of toluol are also added as an antiseptic. The tubes are then removed from the ice and placed in an incubator, kept at a temperature of 40° C., for forty-eight hours. An equal amount of water, roughly enough to fill the test tubes, is added to each test tube and, after shaking up, three drops of iodine solution. The first tube in the descending series which showed a blue or violet colour was taken as the index for comparison.

### QUANTITATIVE DETERMINATION OF THE ACTIVITY OF DIASTASE.

The amount of optically active or reducing sugars produced may be followed polarimetrically or by means of Fehling's solution. Due allowance should be made for any sugar contained in the enzyme.

## PROTEASES.

For many years it has been known that the fluids excreted by many insectivorous plants are capable of digesting proteins; proteolytic ferments are now known to occur in the juice of a good many plants. Some indeed, e.g., erepsin, are almost universal. Amongst the better known ones may be mentioned papaïn which occurs in the fruit of *Carica papaya* (papaw), bromelin in the fruit of the *Ananas sativa* (pine-apple), and cradeïn in the latex and fruit of *Ficus* (fig).

* Appleman: "Bot. Gaz.," 1911, 52, 306.

ISOLATION OF THE ENZYME.

The methods followed in isolating these enzymes differ in details according to the material used; the principle, however, is the same in most cases. The enzyme is precipitated from its solution by reagents, usually alcohol, filtered off, and washed with alcohol. It may be partly purified by dissolving in water and re-precipitating with alcohol. Following are some methods which have been pursued in particular cases.

To isolate the enzymes from the fluid contained within the pitchers of *Nepenthes*, Vines * added to the liquid an equal volume of absolute alcohol, then phosphoric acid followed by lime water in order to increase the bulk of the precipitate. Ammonium carbonate was added until the liquid gave a neutral reaction, and the precipitate filtered off. For use, the precipitate was shaken up with a ·2 per cent solution of hydrochloric acid and filtered; the clear filtrate actively digests fibrin.

If it be desired to examine the contents of a tissue for these ferments, the expressed juice may be used, or an aqueous extract, the enzyme being separated as above if necessary. But sometimes this is unsatisfactory for various reasons—a syrup-like consistency or high coloration, for example. In such cases the tissues may be bruised in a mortar and placed with water in the vessel in which the experiment is to be carried out, together with the material—fibrin, for example—to be acted upon.† Buscalioni and Fermi ‡ used sterilized gelatine, with ·5-1 per cent carbolic acid as an antiseptic, in a Petri dish. Fragments of the tissue to be tested are placed upon the jelly; the liquefaction of the gelatine in the neighbourhood of the pieces indicates the presence of proteolytic enzymes, but inasmuch as all proteases do not attack gelatine, a negative result does not necessarily indicate the absence of these enzymes.

Dean § prepared ereptase from the seeds of beans by extracting the cotyledons with water, filtering, and half saturating the filtrate with ammonium sulphate. The precipitate thus obtained is filtered off, dissolved in water and separated from

* Vines: "Ann. Bot.," 1897, **11**, 573.
† Vines: *id.*, 1903, **17**, 237, 597.
‡ Buscalioni and Fermi: "Ann. R. Inst. Bot. Roma," 1898, **7**, 99.
§ Dean: "Bot. Gaz.," 1905, **39**, 321.

ammonium sulphate by dialysis. The solution of enzyme thus purified may be dried at a temperature below 50° C.

Vines* separated peptase from ereptase by making use of the fact that the former is hardly soluble in water but readily so in a dilute solution of sodium chloride, whilst ereptase is easily soluble in water. The material, e.g. seed of *Cannabis sativa*, is ground and extracted with a 10 per cent solution of sodium chloride. The solution is filtered and rendered just acid by the addition of acetic acid, whereby a white precipitate of protein is formed, which is filtered off. The acid filtrate has marked proteolytic qualities but has no action on fibrin; it therefore contains the ereptase. The fibrin-digesting protease (peptase) is in the precipitate; to recover it, wash the precipitate with a 10 per cent solution of sodium chloride slightly acidified with acetic acid. The precipitate is next treated with distilled water and filtered; the filtrate, which has an opalescent appearance, digests fibrin but has no effect on Witte peptone. In order to ensure the best results, the temperature should be kept as low as possible during filtration.

### GENERAL CONSIDERATIONS.

According to Vines, the proteases of plants fall into two main groups, peptase and ereptase. Peptase hydrolyses proteins to albumose or peptone, but does not act on albumose or peptone whether produced by its own digestion of protein or added in the form of Witte peptone.† Ereptase hydrolyses proteins, albumoses and peptones to amino acids, such as leucine and tyrosine. Peptase dissolves readily in a saline solution but is hardly soluble in water, whilst ereptase is easily soluble in water. Both may occur in a plant, e.g. the seeds of *Cannabis sativa*,‡ the latex of *Carica papaya*—the enzyme of which is termed papaïn—yeast, etc.§ In fact the mixture is, or was, commonly termed vegetable trypsin. On the other hand, some plants which exhibit proteoclastic properties only have peptase. This is, however, seemingly very rare; *Drosera* provides an example.‖

* Vines: "Ann. Bot.," 1908, **22**, 103.
† Vines: *id.*, 1905, **19**, 171; 1908, **22**, 103.
‡ Vines: *id.*, 1908, **22**, 103.
§ Vines: *id.*, 1909, **23**, 1.
‖ White: "Proc. Roy. Soc., Lond.," B., 1910, **83**, 134.

Fisher employs the following terminology: protease, an enzyme which will hydrolyse any protein or the intermediate decomposition products of proteins; proteinase (the peptase of Vines), which will hydrolyse the higher proteins only; and peptase (the ereptase of Vines), which will attack peptones, albumoses, etc.

The proteinases are readily soluble in salt solution, but only slightly soluble in water, or in 50 per cent alcohol. Their activity is greatest at that degree of acidity natural in the plant extract, but pure aqueous extracts show strong activity in neutral or slightly alkaline media, ·05 per cent hydrochloric acid, or ·3 per cent citric acid inhibiting the action.* In this respect they differ from the protease of *Nepenthes*, and from animal pepsin, both of which show their greatest activity in the presence of free acid.

Peptases are readily soluble in water and in aqueous solutions of neutral salts.

Although eraptases are very common in plants, peptases are less common, and have not been found in some cases where they might be expected to obtain, e.g. in protein-containing seeds. Dean's work on *Phaseolus vulgaris* may be taken as an example.† The seeds of this plant contain much protein which undergoes proteolysis before translocation takes place. But no enzyme has been discovered in the seed which is capable of digesting these proteins; ereptase, however, which can hydrolyse the proteases derived from the digestion of these seed proteins, is abundant. Dean considers that the protoplasm plays the part of a peptase, whilst the ereptase may carry the digestion further.

The plant proteases are less rigid than the corresponding ones from animal sources in respect to their activity in acid or alkaline media. Thus the proteolytic enzyme of *Drosera* is active in acid, alkaline or neutral media; papaïn is active both in acid and alkaline media, thus differing from animal pepsin;‡ and some proteases will only work provided the reaction be acid, e.g., *Nepenthes*, malt, mushroom and yeast.§ The natural reaction of the plant juice is the best to maintain for general experiments.

* Fisher: "Biochem. Journ." 1919, **13**, 124.
† Dean: *loc. cit.* ‡ Mendel: "Am. Journ. Med. Sci.," 1902.
§ Vines: "Ann. Bot.," 1905, **19**, 171.

### TRYPTOPHANE REACTION.

The presence of tryptophane is an indication of the activity of trypsin-like proteolytic ferments. Tryptophane may occur naturally in the sap of the plant, its presence being associated with the ripening of fruits and the germination of protein-containing seeds.*

In order to ascertain whether the enzyme be a tryptic one, a solution of it, or some of the more or less crude plant-extract, is added to a solution of peptone and placed in an incubator for some time, according to the strength of the solutions, kept at a temperature of 40°. A little toluol may be added as an antiseptic. To test, a few drops of the liquid are placed in a watch glass, acidified with acetic acid, and then a little chlorine water is added. The appearance of a marked yellow to pink coloration indicates the presence of tryptophane. If performed on a large scale, the liquid may be finally shaken up with amyl alcohol which dissolves the pink chlorine compound and eventually rises to the top. It may be separated by means of a small separating funnel and spectroscopically examined. An absorption band will be observed on the yellow side of the thallium line (571 – 540$\mu\mu$).

Another test for tryptophane consists in mixing the suspected solution with a little glyoxylic acid and carefully adding concentrated sulphuric acid so that the latter forms a separate layer at the bottom of the test tube. After a short time a purple ring is produced at the junction of the two liquids, and on careful agitation the colour extends over the whole solution. If pepsin be used in the above experiments, it must be well washed in water and alcohol before use.

### QUANTITATIVE DETERMINATION OF THE ACTIVITY OF PROTEASES.

Schultz† followed the course of the action of pepsin on egg albumen by precipitating out the albumen from time to time and examining the optical activity of the peptone solution.

Sjöqvist,‡ on the other hand, measured the electrical con-

* Vines: "Ann. Bot.," 1902, **16**, 1; 1903, **17**, 237, 597.
† Schultz: "Zeit. phys. Chem.," 1885, **9**, 577.
‡ Sjöqvist: "Skand. Arch. f. Physiol.," 1895, **5**, 317.

ductivity of an albumen solution which was being hydrolysed by pepsin.*

Sörensen† found that he could obtain a measure of the amount of protein hydrolysed, by determining the number of free carboxyl groups in the mixture. By adding an excess of formalin, the free amino groups were neutralized; the carboxyl groups were then estimated by adding an excess of N/5 baryta solution and titrating back the excess by means of hydrochloric acid.

## ZYMASE AND ALCOHOLIC FERMENTATION.

The formation of alcohol from fluids containing sugar has been known and practised for thousands of years, and the use of yeast in the manufacture of alcoholic beverages and of bread is an ancient industry. As is well known, when yeast is placed in a sugar solution, fermentation begins sooner or later, the principal end products being alcohol and carbon dioxide; substances other than ethyl alcohol, however, are formed, especially glycerol, succinic acid and amyl alcohol,‡ the last more particularly in the fermentation of the sugars obtained from wheat and potato starch. Alcoholic fermentation is due to the activity of the enzyme zymase, which was first separated from the yeast cell by Buchner,§ whose work‖ marks the beginning of an epoch of vigorous investigations into this and kindred subjects.

* Cf. Bayliss: "Arch. Sciences Biol.," St. Petersburg, 1904, 11 (supplem.).

† Sörensen: "Biochem. Zeit.," 1908, 7, 45.

‡ Amyl alcohol, using the term in its general acceptance, is a mixture of two isomeric primary alcohols, isobutyl carbinol $\begin{matrix}CH_3\\CH_3\end{matrix}\!\!>\!CH.CH_2.CH_2OH$ and secondary butyl carbinol $CH_3—CH_2—\underset{\displaystyle CH_3}{\underset{|}{CH}}—CH_2OH$. The two substances together form "fusel oil," which is the harmful constituent of cheap spirit made from potatoes.

They appear to be produced from leucine $(CH_3)_2CH—CH_2—CHNH_2COOH$, and isoleucine $CH_3CH_2—\underset{\displaystyle CH_3}{\underset{|}{CH}}—CHNH_2COOH$, which are constituents of the protein molecule, by loss of $CO_2$ and replacement of the $NH_2$ group by OH (see p. 343). The mixture is optically active owing to the asymmetric carbon atom of the secondary butyl carbinol.

§ For an account of our knowledge of alcoholic fermentation prior to 1897, see Green: "Nature," 21 April, 1898.

‖ Buchner: "Ber. deut. chem. Gesells.," 1897, 30, 117, 1110; 1898, 31, 568; Buchner and Rapp: *id.*, 1897, 30, 2668; 1898, 31, 209, 1084, 1090; 1899, 32, 127.

### THE ISOLATION OF ZYMASE.

The following is the method pursued by Buchner in isolating zymase from *Saccharomyces*. One kilogram of compressed yeast is mixed with 250 grams of the infusorial earth known as kieselguhr and a quantity of fine quartz sand. The mixture is ground in a mortar until the microscope shows the majority of the yeast cells to be broken. To this paste-like mixture are added 100 c.c. of water which is very thoroughly stirred in; the mass is then wrapped in a cloth, placed in a press and gradually subjected to a very high pressure—Buchner used a pressure as high as 500 atmospheres—the liquid extracted being collected in a glass vessel. The residue is then removed from the press, broken up, and again mixed with 100 c.c. of water and subjected to pressure. The extracts are united, shaken up with a little kieselguhr and filtered. The filtrate contains the zymase, but in an impure condition; it may be purified by precipitating with alcohol and dissolving the precipitate in water. The aqueous solution will not keep any great length of time, a character which is shared with most other enzymes when in aqueous solution; this phenomenon is termed hysteresis. It may, however, be preserved for a longer time—but not indefinitely—by drying the extract under reduced pressure, the solid substance so obtained being kept in a cold desiccator and dissolved in water as occasion demands.

In preparing extracts of yeast, it must be remembered that the potency of the extracts depends upon the physiological state of the yeast used. Thus, if brewers' yeast be taken from the wort whilst fermentation is at its height, a high quality zymase will be obtained; if, however, fermentation of the wort be over, the yeast taken from it will yield an extract of little or no fermenting power.

### GENERAL CONSIDERATIONS.

The alcoholic fermentation of sugar by yeast may be represented by the equation:—

$$C_6H_{12}O_6 = 2CO_2 + 2C_2H_6O$$

Recent investigations, however, have shown that the phenomenon is not quite so simple as this general statement indicates.

The names of Harden and Young particularly are associated with the problem, and a brief account of their work may be given.*

The alcoholic fermentation of glucose by yeast-extract is greatly increased by the addition to the fermenting liquor of yeast-juice, which has been boiled—so that all enzymes are destroyed—and filtered. After such addition, there is to begin with a greater evolution of carbon dioxide, which gradually diminishes to a rate which remains practically constant for several hours and usually is about equal to that given by an equal volume of the same yeast-extract and glucose to which boiled and filtered extract has not been added; but the diminution in the fermentation rate is slower than in the control, so that fermentation continues for a longer period, the extra amount of carbon dioxide evolved being directly proportional to the volume of boiled juice added. The accelerating factor is a phosphate, and analysis showed that the extra quantity of carbon dioxide evolved in the initial period of high evolution, when a phosphate or boiled extract is added, corresponds to the evolution of one molecular proportion of carbon dioxide for each atom of phosphorus added in the shape of phosphate. It should thus be possible to separate yeast-juice into two complementary parts, either one of which depends upon the presence of the other in order that fermentation may take place. This was accomplished by filtering the expressed yeast-juice through a Martin gelatine filter under a pressure of 50 atmospheres. The residue remaining in the candle is inactive,† and so also is the filtrate which contains the active principle, which is neither destroyed by heat nor precipitated by ammoniacal magnesia mixture. This activating substance, or co-ferment, is a salt of hexose phosphoric acid.

The alcoholic fermentation of glucose therefore takes place

* Harden and Young: "Proc. Roy. Soc., Lond.," B., 1906, **77**, 405; 1906, **73**, 369; 1908, **80**, 299; 1909, **81**, 336; 1910, **82**, 321; 1911, **83**, 451; "Centrlbl. Bakt.," 1910, **26**, 178. Young: "Proc. Roy. Soc., Lond.," B., 1909, **81**, 523; "Biochem. Zeit.," 1911, **32**, 177.

† The aqueous solution of the residue does not keep for long; it may, however, be preserved for some time by spreading it out on a clock glass and placing in a sulphuric acid desiccator. It dries to a brown, brittle substance which can easily be ground to a powder. In order to purify, the powder may be dissolved in water and once more filtered and dried as before.

in stages, the first of which is the formation of hexose phosphate which takes place during the first period of temporary acceleration.

(1) $2C_6H_{12}O_6 + 2R_2HPO_4 = 2CO_2 + 2C_2H_6O + C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O$

This reaction only takes place provided that the enzyme and the co-ferment are present; soluble phosphates alone are unable to promote the fermentation in a mixture of the enzyme and glucose.

The hexose phosphate is continually being hydrolysed by an enzyme, hexose-phosphatase, yielding a free phosphate which again enters into combination with hexose:—

$$C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O = C_6H_{12}O_6 + 2R_2HPO_4$$

The rate at which this second reaction takes place is the determinating factor in the fermentation rate when glucose is fermented by yeast-extract. There is an optimum concentration of phosphate which produces a maximum initial rate of fermentation; beyond this optimum a further addition of phosphate depresses the fermentative activity. If the available amount of phosphate in a mixture of sugar, ferment and co-ferment be very small, the total fermentation is greatly reduced, but if to such a mixture a little phosphate be added, there is an enormous increase, as much as 700 per cent, in the total fermentation, even after discounting an amount of carbon dioxide equivalent to the phosphate added.

With regard to other sugars, Harden and Young found that mannose and fructose are freely fermented by yeast-extract, fructose being fermented more quickly than mannose and mannose rather more quickly than glucose. Also the total weight of carbon dioxide given off from an excess of sugar by the action of a given volume of yeast-juice was slightly greater with fructose than with glucose, whilst that evolved from mannose was less than from glucose. No matter what sugar is used, glucose, fructose or mannose, the hexose phosphate is the same. The behaviour of fructose is qualitatively the same as glucose, but quantitatively there is a considerable difference. Thus the optimum concentration of phosphate for the fermentation of fructose is from 1·5 to 10 times as great as the optimum for glucose, and the maximum rate of fermentation of fructose is 2 to 6 times as great as that of glucose.

Fructose also behaves in the presence of other sugars as

an accelerating factor, for if the rate of fermentation of glucose or of mannose by yeast-extract is greatly lowered by the presence of a large excess of phosphate, the addition of a relatively small quantity of fructose brings about a marked acceleration in the fermentation. This is not due solely to the fermentation of the added fructose, for the amount of carbon dioxide evolved is much too great. This appears to be a specific property of fructose, for the phenomenon does not obtain when glucose is added to mannose or fructose, or by mannose when added to glucose or fructose under the proper conditions of concentration of phosphate in each case.

Commenting on this, Harden and Young observe that "this remarkable property of fructose, taken in connexion with the facts that this sugar in the presence of phosphate is much more rapidly fermented than glucose or mannose, and that the optimum concentration of phosphate for fructose is much higher than for glucose or mannose, appears to indicate that fructose when added to yeast-juice does not merely act as a substance to be fermented, but, in addition, bears some specific relation to the fermenting complex".

It is supposed that fructose forms a permanent part of the fermenting complex, so that a greater concentration of this sugar in yeast-extract leads to the formation of an increased quantity of complex. Thus, owing to the increased concentration of this active catalyst, the yeast-juice could bring about the reaction with sugar in the presence of phosphate at a higher rate and, at the same time, the optimum concentration of phosphate would become greater.

Harden and Young also find that the addition of a suitable amount of arsenate to a fermenting mixture of yeast-extract and sugar (glucose, fructose or mannose) causes a marked acceleration in the rate of production of alcohol and carbon dioxide, which is continued long after a chemical equivalent of carbon dioxide has been evolved. In this, the action of arsenate differs from that of phosphate and, further, the arsenate occurs in the free state throughout the period of fermentation. This increased rate of fermentation is due to the accelerating influence of the arsenate on the hexose-phosphatase; the arsenate, however, cannot replace phosphate in the fundamental reactions of alcoholic fermentation.

That phosphate is a necessity for alcoholic fermentation by zymase is generally agreed, but views other than the above have been put forward regarding the part played by it in fermentation.

Iwanoff,* for instance, considers that the phosphate formed is a triose phosphate, the formation of which is not necessarily accompanied by the evolution of carbon dioxide and alcohol, since the combination will take place when a phosphate is added to the filtrate of a solution of sugar which has been fermented by yeast-extract. He also found that the sugar obtained from the sugar phosphate is not fermented by living yeast. Iwanoff concludes that there are three stages in alcoholic fermentation: the sugar is first broken down into simpler sugars, then by the action of an enzyme, termed synthease, a triose phosphate is organized, which is then acted upon by alcoholase to form carbon dioxide, etc.

These views are not agreed with by Harden and Young,† who criticize the methods employed by Iwanoff.

In addition to phosphate, other substances may act as co-enzymes: thus Neuberg ‡ finds that aldehydes generally accelerate the alcoholic fermentation of dextrose and mannose, and that a mixture of keto acids with potassium phosphate acts as a coenzyme.

The equations given above for the formation of hexose phosphate, whilst representing an important preliminary reaction, do not throw any light on the subsequent changes involved in the formation of the alcohol, carbon dioxide, glycerol, and acetic aldehyde from the sugar.§ Many theories have been put forward to explain the mechanism of alcoholic fermentation; the more recent views of Neuberg and Reinfurth‖ appear to be well supported by the facts of experiment. The first action would appear to be a molecular rearrangement of the glucose molecule with the formation of one molecule of glyceric acid and one of dihydroxyacetone.

* Iwanoff: "Centrlbl. Bakt.," 1909, **24**, 1

† Harden and Young: *id.*, 1910, **26**, 178.

‡ Neuberg: "Biochem. Zeitsch.," 1918, **88**, 145.

§ The amyl alcohol and succinic acid, which also are found amongst the products of alcoholic fermentation, are produced not from the sugar, but from the protein present in the yeast cell (see p. 343).

‖ Neuberg and Reinfurth: "Ber. deut. chem. Gesells.," 1919, **52**, B, 1677.

$$CH_2OH.CHOH.CHOH.CHOH.CHOH.CHO =$$
$$CH_2OH.CHOH.CHO + CH_2OH.CO.CH_2OH$$
glyceric aldehyde — dihydroxyacetone

The two latter compounds by the loss of one molecule of water would each yield methyl-glyoxal.

$$\left.\begin{matrix} CH_2OH.CHOH.CHO - H_2O \\ CH_2OH.CO.CH_2OH - H_2O \end{matrix}\right\} CH_3CO.CHO$$
methyl-glyoxal

From these two molecules of methyl-glycoxal, molecular proportions of glycerol and pyruvic acid would result from the simultaneous oxydizing and reducing and hydrolytic action of two molecules of water.

$$\begin{matrix} CH_2=COH.CHO \\ CH_3.CO.CHO \end{matrix} + \begin{matrix} H_2 \\ \| \\ O \end{matrix} = \begin{matrix} CH_2OH.CHOH.CH_2OH \\ + \\ CH_3.CO.COOH \\ \text{pyruvic acid} \end{matrix}$$

The pyruvic acid is then acted upon by a carboxylase in the yeast yielding acetic aldehyde and carbon dioxide.

$$CH_3.CO.COOH. = CH_3CHO + CO_2$$

Another molecule of water simultaneously reduces the aldehyde to ethyl alcohol, and oxidizes a fresh molecule of methyl-glycoxal to pyruvic acid.

$$\begin{matrix} CH_3.CHO \\ CH_3.CO.CHO \end{matrix} + \begin{matrix} H_2 \\ \| \\ O \end{matrix} = \begin{matrix} CH_3CH_2OH \\ + \\ CH_3.CO.COOH \end{matrix}$$

It was found by these same authors * that if sodium sulphite were added to the fermenting mixture, so that the acetic aldehyde formed as above is fixed at once in the form of its bisulphite addition compound, it is not further reduced, and the hydrogen which thus becomes avaible is used in producing glycerol by the reduction, presumably, of the glyceric aldehyde. According to theory, a glucose molecule could yield one molecule of glycerol and one molecule of acetaldehyde:—

$$C_6H_{12}O_6 + Na_2SO_3 + H_2O = C_3H_8O_3 + CH_3.CHOH.SO_3Na + HNaCO_3$$

About 70 per cent of this theoretical yield of glycerol was obtained, which corresponds to about 35 per cent of the glucose employed.

* Neuberg and Reinfurth: "Biochem. Zeitsch.," 1918, 89, 365.

This discovery of the effect of adding sulphites to the fermenting mixture assumed technical importance in Central Europe during the great shortage of fats. The yield of glycerol obtained by this process amounted in some cases to over 20 per cent and by employing concentrations of sodium sulphite equivalent to 200 per cent of the sugar concentration, a yield of 36·7 per cent was obtained.*

According to Buchner, lactic acid is an intermediate product of fermentation; in the first place the glucose under the influence of zymase is converted into lactic acid, which is then attacked by another enzyme, the action giving origin to carbon dioxide and alcohol.

Kohl,† however, points out that lactic acid is not fermented by zymase, by compressed yeast nor by bottom yeast; indeed 1 per cent lactic acid is sufficient to stop the auto-fermentation of yeast and to reduce greatly the fermentation of glucose. On the other hand, zymase will ferment sodium lactate, which indicates that if lactic acid is an intermediate product of fermentation, according to Buchner's view, a salt rather than the acid must be formed.

In yeast-extract Kohl found an enzyme, catalase, which was capable of oxidizing phenols. The yeast-extract on filtering produces lactic acid in the presence of glucose, and the acid in the presence of zymase is converted into alcohol and carbon dioxide; if, however, zymase be not present, oxidation may go further and other acids be produced.

Briefly put, he considers that the glucose, by the action of catalase, is converted into lactic acid which is operated upon by zymase, so that alcohol and carbon dioxide are produced.

Lebedev‡ considers that the balance of evidence is not favourable to the view that lactic acid is an intermediate product; he considers that pyruvic aldehyde is more likely, although the evidence is not conclusive, since at low concentrations it is decomposed by yeast to practically equivalent amounts of alcohol and carbon dioxide.

Zymase-like enzymes are not restricted to the yeasts; such

* Connstein and Lüdecke: "Ber. deut. chem. Gesells.," 1919, **52**, 1385. See also Zerner: "Ber. deut. chem. Gesells.," 1920, **53**, [B], 325.

† Kohl: "Beih. bot. Centrlbl.," 1910, **29**, 115.

‡ Lebedev: "Biochem. Journ.," 1917, **11**, 189.

bodies have been identified in other Fungi, such as *Mucor stolonifera* * and *Aspergillus niger*.†

### OTHER ENZYMIC ACTIVITIES OF SACCHAROMYCES.

In addition to zymase, other enzymes are associated with yeast, e.g. diastase, invertase, trypsin, protease, emulsin, and peroxidase.

For laboratory purposes the fermentative activity of *Saccharomyces* may be quickly and conveniently illustrated by the use of Pasteur's solution, the composition of which is as follows:—

Ammonium tartrate, 50 grams
Potassium phosphate, 10 grams
Calcium phosphate, 1 gram
Magnesium sulphate, 1 gram

These salts are thoroughly ground and mixed in a mortar, and 1 gram of the mixture together with 12 grams of glucose are dissolved in 70 c.c. of water, the yeast being added to the solution.

If cane sugar be used, marked fermentation will only begin after an interval of time has elapsed, during which the invertase secreted by the yeast converts the sucrose, which is not directly fermentable by yeast, into invert sugar.

But, according to Bokorny,‡ sucrose is better than glucose, and urea is better than ammonia in the culture solution. Access of air is important, but the presence or absence of light does not much matter.

Yeast, or yeast-juice, can set up fermentation in other substances besides glucose, such, for example, as galactose,§ mannose, fructose,‖ sodium lactate,¶ and, according to Neuberg and Tir,** common plant acids, fatty acids, glycerol, and lecithin.

* Kostytschew: "Ber. deut. bot. Gesells.," 1904, **22**, 207.
† Maximow: *id.*, 1904, **22**, 225.
‡ Bokorny: "Allg. Brau. Hopfen. Zeitsch.," 1917, **57**, 447.
§ Harden and Norris: "Proc, Roy. Soc., Lond.," B., 1910, **82**, 645.
‖ Harden and Young: *id.*, 1909, **81**, 336.
¶ Kohl: "Beih. bot. Centrlbl.," 1910, **29**, 115.
** Neuberg and Tir: "Biochem. Zeitschr.," 1911, **32**, 323.

This fermentation, however, may not take place immediately on the introduction of the yeast to the particular substance: for instance, before yeast can ferment galactose, it must be educated with regard to this material by being cultivated for some time in a solution containing it. A yeast so educated yields a juice which can ferment galactose, the fermenting mixture, according to Harden and Norris, reacting with phosphate in a manner exactly similar to yeast-extract and glucose; further, the process is accelerated by the addition of a small quantity of sodium arsenite.

In addition to ordinary alcoholic fermentation, yeast also exhibits the power of auto-fermentation.* This is brought about at the expense of the reserve food materials of the plant, chiefly glycogen, two enzymes being concerned in the process. Glycogenase changes the glycogen into sugar, which is then converted by zymase into alcohol and carbon dioxide, the rate of fermentation being dependent on the rate of sugar production by the glycogenase. Harden and Paine also found that the rate of auto-fermentation is greatly increased by the removal of water from the cell, which means, of course, a concentration of the cell-sap. This may be accomplished by partial desiccation or by the use of dissolved substances which plasmolyse the cells. Alcohol in solutions above 10 per cent also have the same effect; on the other hand, salts which do not produce plasmolysis, even in concentrated solutions, such as urea, have no such accelerating effect.

### OCCURRENCE OF ALCOHOLS IN PLANTS.

*Methyl Alcohol* has been found to occur in the aqueous distillates and in the essential oils of a very large number of different plants, amongst which might be mentioned *Juniperus Sabina*, *Zea Mais*, *Lolium perenne*, *Iris germanica*, *Euonymus europaea*, *Thea sinensis*, *Eugenia caryophyllata*, *Carum carvi*, *Anthriscus cerefolium*, etc.

*Ethyl Alcohol* is not quite so widely distributed as methyl alcohol, but occurs in distillates from *Cananga odorata* (Ylang Ylang), *Pyrus Malus*, *Mespilus germanica*, *Eucalyptus*, *Anthriscus cerefolium*, *Pastinaca sativa*, *Vaccinium Myrtillus*, *Betula alba*, etc.

* Harden and Paine: "Proc. Roy. Soc., Lond.," B., 1912, 84, 44.

Mention also should be made of the occurrence of this alcohol, together with lactic acid and acetone* in some cases, in the higher plants especially during anærobic respiration. Stoklasa,† for instance, found that this substance together with acetic and formic acids was produced during anærobic respiration of potatoes and seeds. Indeed, many consider that alcoholic fermentation is the first expression of respiration, and whether alcohol is formed or not depends upon the conditions; thus under normal conditions in the presence of oxygen the first products are oxidized before the alcohol stage in the process is reached, or the alcohol may be used up in anabolic processes as soon as it is formed, or it may be oxidized to water and carbon dioxide—the normal end products of ærobic respiration.‡

*Amyl Alcohol* has been identified in the essential oils of geranium, eucalyptus, lavender, peppermint and chamomile.

Several unsaturated alcohols, such as citronellol $C_{10}H_{20}O$, geraniol and linalool, both of the formula $C_{10}H_{18}O$, occur in essential oils, such as rose oil and oil of bergamot, while amongst the alcohols belonging to the aromatic series must be mentioned cinnamic alcohol, benzyl alcohol, menthol, borneol, etc. Other monohydric alcohols, with the exception of phytosterol (see p. 18), are of comparatively rare occurrence.

Examples of polyhydric alcohols occurring in plants are mannitol, sorbitol, and dulcitol, isomeric substances of the formula—

$$CH_2OH\ CHOH\ CHOH\ CHOH\ CHOH\ CH_2OH$$

*Mannitol* occurs to the extent of about 40-50 per cent in manna, the dried sap of *Fraxinus Ornus*, and also in celery, *Syringa vulgaris*, asparagus, cauliflower, carrot, pulse, etc. *Sorbitol* occurs in the berries of the mountain ash, *Pyrus Aucuparia*. *Dulcitol* occurs in the cortex of *Euonymus europaea* and in the bark of *Euonymus atropurpurea*.

*Adonitol*,§ $C_5H_{12}O_5$ or

$$CH_2OH\ CHOH\ CHOH\ CHOH\ CH_2OH,$$

* Palladin and Kostytschew: "Ber. deut. bot. Gesells.," 1906, **24**, 273.

†Stoklasa: *id.*, 1904, **22**, 358; "Centr. f. Bakter. u. Parasit.," 1905, II, **31**, 86. Godlewski and Polzeniusz: "Bull. Acad. Sci., Cracow," 1901, 227; Stoklasa, Jelinek and Vitek: "Beitr. z. chem. Phys. u. Path.," 1903, **3**, 460.

‡ See Kostytschew: "Ber. deut. bot. Gesells.," 1908, **26**, 565.

§ Merk: "Chem. Zentr.," 1893, 344.

is a pentahydric alcohol occurring in *Adonis vernalis*. According to Treboux,* it is converted by the plant into starch. Adonitol has a sweet taste, and is used in bacteriological media.

Of recent years a number of dihydric alcohols of high molecular weight have been found to occur in plants. They belong to different series whose general formulæ are—

$$C_nH_{2n-6}O_4,\ C_nH_{2n-8}O_4,\ \text{and}\ C_nH_{2n-10}O_4.$$

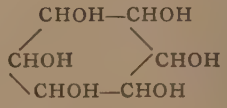
*Trifolianol*, $C_{21}H_{34}O_2(OH)_2$, isolated by Power and Salway,† from red clover leaves, may be taken as an example of the first group, while *Bryonol*, $C_{22}H_{34}O_2(OH)_2$, obtained by Power and Moore ‡ from Bryony root, and *Calabarol*, $C_{23}H_{34}O_2(OH)_2$, isolated by Salway § from Calabar beans, are representatives of the second and third groups respectively.

Of the polyhydric alcohols Inositol is of particular interest, and may, therefore, receive more detailed consideration.

## INOSITOL.

Inositol, which has the formula $C_6H_{12}O_6$, is isomeric with the hexoses, and, like these substances, has a sweet taste; for these reasons, it was at one time thought to be a true sugar and was called muscle sugar owing to its occurring in muscle.

Inositol is, however, not a carbohydrate at all but a polyhydric alcohol derived from benzene and having the constitution—




Besides being found in muscle, inositol is of common occurrence in plants, in the leaves, especially when young, of *Vitis*, *Juglans*, etc.; in the roots and rhizomes of very many plants; in various seeds and fruits, e.g. *Phaseolus*, *Pisum*, and other leguminous seeds, *Vitis*, various cereals, and oily seeds, such as mustard.

* Treboux: "Ber. deut. bot. Gesells.," 1909, **27**, 428.
† Power and Salway: "J. Chem. Soc., Lond.," 1910, **97**, 249.
‡ Power and Moore: *id.*, 1911, **99**, 943.
§ Salway: *id.*, 1911, **99**, 2155.

It may be looked upon as a plastic substance since Maquenne has found that it disappears from the young fruits of *Phaseolus* as ripening proceeds.

*Preparation.*

The method of separation of inositol from the plant juices is based on the fact that it forms a compound with lead oxide.

The sap is expressed from the organ, or, if this be impracticable, the parts are ground up very thoroughly with water. The liquid is then filtered and, if it gives an acid reaction, is neutralized by the addition of baryta water.

A solution of basic lead acetate is then added until no more precipitate comes down. The precipitate consists of a compound of inositol with lead oxide ($2C_6H_{12}O_6$ $5PbO$), and is filtered off, then washed and suspended in water, and saturated with a current of sulphuretted hydrogen. The lead sulphide is filtered off and the filtrate evaporated on a water bath to the consistency of a syrup. On the addition of alcohol, containing one-tenth of its volume of ether, inositol is deposited in prismatic crystals.

Inositol has a sweet taste, is soluble in water but insoluble in alcohol and ether. It crystallizes in prisms, and does not give any reactions characteristic of true hexoses. For instance, it is not fermentable, it does not reduce Fehling's solution, and its solution does not give a brown coloration with potash.

*Identification.*

1. When moistened with a little dilute nitric acid, then evaporated almost to dryness, and made alkaline with ammonia, the addition of a few drops of chloride of calcium produces a rose-red coloration.

2. A solution of inositol evaporated to dryness with a few drops of mercuric nitrate produces a yellow stain which on heating turns red.

3. Solutions of inositol are not optically active.

With regard to its significance in the plant there is evidence to show that inositol is a transitory substance and is used up in the synthesis of other substances.

According to Posternack,* a large amount, 80-90 per cent, of the phosphorus of certain seeds exists in the form of phytin; it occurs, for instance, in the globoid portion of aleurone grains, and the seeds which contain it also possess an appropriate enzyme phytase for its decomposition into phosphoric acid and inositol.†

With regard to the formation of phytin little is known; Posternack considers that it is formed by the combination of formaldehyde, produced in the early stages of photosynthesis with phosphoric acid.

The tenability of this opinion is obviously bound up with the formation of formaldehyde in green leaves (q.v.).

Phytin appears to be an acid calcium and magnesium salt of inositol phosphoric acid which is a condensation compound of inositol with six molecules of phosphoric acid.‡

### MANUFACTURE OF ETHYL ALCOHOL.

The action of yeast on sugar is made use of in the manufacture of ethyl alcohol, which substance is prepared from potatoes, rice, and other grains rich in starch. The manufacture from potatoes is carried out as follows: Potatoes are heated in closed vessels to 125-135° by means of superheated steam under a pressure of about 3 atmospheres; by suddenly releasing the pressure the potatoes are burst, and are thus obtained in a finely divided state. The whole mass is then thoroughly stirred up with malt at a temperature of about 60°, whereby the starch undergoes hydrolysis with formation of maltose and dextrin.

$$\underset{\text{Starch}}{(C_6H_{10}O_5)_n} + H_2O \rightarrow \underset{\text{Maltose}}{C_{12}H_{22}O_{11}} + \underset{\text{Dextrin}}{(C_6H_{10}O_5)_x}$$

After about one and a half hours the mixture is rapidly cooled to 15° and mixed with yeast; fermentation at once sets in, accompanied by a considerable evolution of heat; the

* Posternack: "Compt. rend.," 1903, **137**, 202, 337, 439.

† Cf. Suzuki, Yoshimura and Takaishi: "Bull. Coll. Agric., Tokyo," 1907, **7**, 503. See also Rose: "Biochem. Bull.," 1912, **1**, 428.

‡ Cf. Neuberg: "Biochem. Zeitschr.," 1908, **9**, 557; Winterstein: "Zeitschr. physiol. Chem.," 1908, **50**, 118. See also Plimmer: "Biochem. Journ.," 1913, **7**, 43; Boutwell: "Journ. Amer. Chem. Soc.," 1917, **39**, 491; Posternack: "Compt. rend.," 1919, **169**, 37, 138.

mixture is therefore cooled artificially, so that the temperature is maintained steady at about 27°·5-30°.

During this time the maltose is converted first into dextrose and then into alcohol and carbon dioxide according to the equations :—

$$C_{12}H_{22}O_{11} + H_2O = 2C_6H_{12}O_6$$
$$C_6H_{12}O_6 = 2C_2H_5OH + 2CO_2$$

In order to convert the dextrin, which would otherwise be lost, into a fermentable substance, the temperature towards the end is maintained at about 26-29° in order to give the malt a further opportunity of hydrolysing the dextrin to glucose, and so rendering it capable of being fermented by yeast. When the fermentation is completed after about three days, the mixture contains about 13 per cent of alcohol by volume; by distilling the mixture through a fractionating column, so much of the water is removed that the distillate contains about 80 to 95 per cent of alcohol.*

No amount of fractional distillation without dehydrating agents will produce alcohol containing less than 4·43 per cent by weight of water, since such alcohol gives a constant boiling mixture.

Alcohol containing 0·5 per cent or less of water is, in commerce, known as *absolute alcohol*, although in a scientific laboratory the term is only correctly applied to alcohol which is quite free from moisture; such alcohol can only be obtained by careful fractionation from freshly burnt quicklime.† If the alcohol is dehydrated over quicklime to which a little barium oxide has been added, complete dehydration is marked by the formation of a yellow colour due to the production of barium ethylate, which can only be formed in the absence of any trace of moisture.

A delicate test for the detection of traces of moisture in alcohol consists in adding a few drops of the sample to a solution of liquid paraffin in anhydrous chloroform; if there is any moisture present, a turbidity will be at once produced.

* The residue remaining after distillation contains, in addition to the solid unfermentable materials, a certain amount of other soluble products of fermentation, such as glycerol and succinic acid; it is used as a cattle food.

† Occasionally the last traces of moisture are removed by treating the alcohol with sodium wire.

## OXIDASES.

The oxidases are enzymes which have the power of oxidizing various aromatic compounds and chromogens, which action is frequently indicated by a change in colour. This change in colour in vegetable tissues on exposure to air is an everyday phenomenon; the exposed surfaces of a bitten apple, especially cider varieties, will rapidly turn brown; similarly the fruit-body of *Boletus* quickly assumes a Prussian-blue colour on being broken. The darkening in the colour of raw rubber is also due to an oxidase which is associated with the protein of the coagulated latex.*

These changes are often of considerable economic importance; thus the discoloration of sap wood markedly depreciates the value of the timber,† while the lacquer industry of China and Japan has been built up on the facts relating to the action of the oxidase, laccase, on the expressed sap of species of *Rhus*. (See below.)

Oxidases are very widely distributed in the vegetable kingdom; in the higher plants they may occur in any organ —stem, root, leaf, laticiferous tissue, petals, and fruits.

Several oxidases have been distinguished, e.g. laccase, which has already been mentioned; tyrosinase, which oxidizes tyrosine into homogentisic acid (p. 361); olease, from olives, which can oxidize fats into simpler fatty acids,‡ and others which oxidize sugars into carbon dioxide and water.§

The action of oxidases may be illustrated by a brief reference to laccase, an enzyme which was first investigated by Yoshida.‖ The latex of many species of *Rhus* rapidly turns brown and finally black on exposure to the atmosphere; if the juice be evenly spread out, the final product is black and shiny. The extract of the plant contains urushic acid (laccol) which is oxidized into oxyurushic acid:—

$$C_{14}H_{18}O_2 + O = C_{14}H_{18}O_3$$

The action takes place best at 20° C. in the presence of moisture and oxygen; at higher temperatures it is destroyed, at

* Spence: "Biochem. Journ.," 1908, **3**, 165, 351.
† Bailey: "Bot. Gaz.," 1910, **50**, 142.
‡ Tolomei: "Chem. Centrbl.," 1896, **1**, 879.
§ See Palladin: "Zeit. physiol. Chem.," 1906, **47**, 407.
‖ Yoshida: "J. Chem. Soc., Lond.," 1883, **43**, 472.

63° according to Yoshida, and at 70° according to Bertrand. Bertrand * also has given much attention to this oxidase, and the most important fact ascertained by him in this connexion is that the presence of manganese is all-important. He found that the activity of the ferment is directly proportional to the amount of the metal present, which acts as a co-enzyme (p. 357). But whether manganese is essential for all oxidase reactions is uncertain, for Bach † states that he has prepared a tyrosinase which will oxidize tyrosine in the absence of manganese and of iron.

### ISOLATION OF OXIDASES.

The isolation of oxidase may be a difficult matter when it exists in a tissue together with its substrate and other enzymes. Bourquelot and Bertrand give the following method for Fungi such as *Russula*. The tissue is chopped up, extracted with water—which may be warmed—and filtered as quickly as may be. The filtrate is then poured into an excess of strong alcohol, whereby the enzyme is precipitated. The precipitate is then filtered off and dissolved in water.

When the oxidase is extracted together with other oxidizing enzymes, separation may be effected, at any rate in part, by adding to the aqueous solution of the oxidase, or to the aqueous extract of the plant, two volumes of absolute alcohol for each volume of extract. The oxidase will be precipitated, whilst the other enzymes will remain in solution. ‡

### PEROXIDASE.

Peroxidases which split oxygen from hydrogen peroxide, organic peroxides, potassium permanganate, etc., are often associated with other oxidases, and are very widely distributed; indeed, they have even been described as occurring in coal. §

According to Willstätter and Stoll, ‖ the peroxidase of the horse-radish appears to be a nitrogenous glucoside containing

* Bertrand: "Compt. rend.," 1895, **120**, 266; 1895, **121**, 166; 1896, **122**, 1132; 1896, **123**, 463; 1897, **124**, 1032, 1355.

† Bach: "Ber. deut. chem. Gesells.," 1910, **43**, 362.

‡ Aso: "Bull. Coll. Agric. Imp. Univ., Tokyo;" 1902, **5**, 207.

§ Stoklasa, Ernst, and Chocensky: "Ber. deut. bot. Gesells.," 1907, **25**, 38.

‖ Willstätter and Stoll: "Annalen," 1918, **416**, 21.

over 30 per cent of a pentose and an equimolecular proportion of a hexose; if it contains only two sugar molecules its molecular weight would appear to be about 500 with three atoms of nitrogen; it contains 5·5 per cent of ash, and 0·46 per cent of iron. Associated with it is an impurity, also a introgenous glucoside, of higher molecular weight which gives Millon's and xanthoproteic reactions; it contains about 50 per cent of pentose, a hexose residue, and three atoms of nitrogen to every two molecules of pentose.

### *Preparation of Peroxidase.*

Appleman* recommends the following method of preparing peroxidase. Potato tubers are grated into a pulp, which is thoroughly mixed with calcium carbonate in order to neutralize any acids. The mass is then ground with quartz sand in a mortar for about two minutes and filtered through butter muslin. The extract contains the peroxidase together with oxidase; the latter may be removed by raising the temperature to 70° for ten minutes, whereby the oxidases are coagulated, or, according to Gruess,† any oxidase present may be destroyed by adding acetone which does not affect the peroxidase. In many experiments where only a dilute solution of peroxidase is required—1 c.c. of extract to 300 c.c. of water—the heating may be dispensed with, as the amount of oxidase is so very small.

The method followed by Gruess‡ is somewhat different. The potatoes are sliced into absolute alcohol, and the oxidases destroyed by heating to 70° for ten minutes. The slices are allowed to remain twenty-four hours in absolute alcohol, which should be changed at least three times. The material is then superficially dried with filter paper and covered with ether for a few minutes. The dehydrated slices are then freed from the alcohol and ether by placing in a vacuum desiccator, after which they may be ground up in a mortar. Before use, 1 gram of the powder is ground with sand and 25 c.c. of water, and then filtered.

This method is criticized by Appleman, who points out that in the process of drying, the activity of the peroxidase is

* Appleman: "Bot. Gaz.," 1911, **52**, 306.
† Gruess: "Ber. deut. bot. Gesells.," 1903, **21**, 356.
‡ Gruess: "Zeit. Pflanzenkrank," 1910, **25**, 115.

greatly impaired, and also that the presence of coagulable proteins interfere with the stability of the peroxidase activity, besides causing a low yield of enzyme.

Willstätter and Stoll * have elaborated a process of extraction which in outline is as follows: Five kilos of finely sliced horse-radish are first washed for some days in running water, to remove soluble substances by dialysis through the cell membranes; the material is then warmed for a few hours with a 0·4 per cent solution of oxalic acid, whereby the enzyme is precipitated; the residue is next crushed and washed on a filter with 15 litres of very dilute oxalic acid; it is then ground up with baryta water to liberate the enzyme and the expressed liquid is treated with carbon dioxide to precipitate the barium. The addition of alcohol precipitates slimy substances which are removed and the filtrate is then evaporated to 50 c.c.; the addition of five times this bulk of alcohol will precipitate the crude enzyme; the latter is purified by solution in water containing a trace of sulphuric acid and reprecipitation with alcohol. The resulting product is a mixture of the enzyme with a nitrogenous glucoside; the latter forms an insoluble compound with mercuric chloride, and is precipitated out of solution by this means and the filtrate is once more precipitated with alcohol; after several more solutions and reprecipitations, the enzyme is obtained free from adhering glucoside.

The best yield of enzyme from 5 kilos of horse-radish was 0·45 gram, which was about 60 per cent of the total enzyme present.

### *Identification.*

1. Guaiacum tincture in the presence of oxidase turns blue provided oxygen be present.

2. In cases where the blueing of the guaiacum tincture does not take place immediately, the addition of hydrogen peroxide may bring it about.

3. Tetramethyl-*p*-phenylenediamine in the presence of hydrogen peroxide gives a deep violet colour with an oxidase.

* Willstätter and Stoll: "Annalen," 1918, **416**, 21.

4. Peroxidases set free oxygen from hydrogen peroxide and other peroxides.

For comparative experiments with peroxidase, Gruess uses 1 gram of pulverized powder, prepared as above, which is mixed and ground with sand with 25 c.c. of water. For the test, 5 c.c. of the filtrate is mixed with ·5 c.c. of guaiaconic acid dissolved in alcohol, and ·1 c.c. of a ·5 per cent solution of hydrogen peroxide.

Appleman, for comparative tests, allows a definite quantity of the extract (see above) to act on a definite volume of guaiaconic acid solution in the presence of hydrogen peroxide, the test tube being kept at a constant temperature whilst the experiment is going on. For the comparison, a standard blue aqueous solution of indigo carmine is made; the time required for the blue of the guaiacum mixture to match the colour of the standard blue is taken as the index of the peroxidasic activity.

It should be remarked that, according to Aso, the presence of certain substances, e.g., tannin or sodium fluoride, interferes with the colour reactions normally given by oxidases.

*Estimation.*

Willstätter and Stoll have elaborated a method, depending on the production of purpurogallin from pyrogallol and hydrogen peroxide in the presence of the peroxidase, for estimating peroxidase. It is termed the "purpurogallin number," and represents the number of milligrams of purpurogallin which would be produced by 1 mgm. of the vacuum-dried preparation. This number is about 0·25 for well-pounded horse-radish, 360 for the crude enzyme, before purification by means of mercuric chloride, and about 670 for the purest sample of the enzyme so far obtained.

GENERAL CONSIDERATIONS.

Up to comparatively recent times an oxidase was considered to be a single enzyme, but according to Bach and Chodat,* what used to be termed oxidase is really a mixture

* Bach and Chodat: "Biochem. Centrbl.," 1903, 1, 416; Bach: "Ber. deut. chem. Gesells.," 1906, 39, 2126; 1907, 40, 230; 1908, 41, 216.

of peroxidase and peroxide. According to them, there are three categories of oxidizing ferments.

(*a*) Oxygenases which produce the peroxide.

(*b*) Peroxidases which transfer oxygen from the peroxide to the substance to be oxidized.

(*c*) Catalases which destroy peroxides so that oxygen is given off.

In the colour reactions mentioned above two actions are possible. Either the plant juice, e.g. of the potato, gives the blue coloration with the guaiacum tincture alone, or, the blue colour will not occur, as, for example, in the sap of the cucumber, unless a peroxide, such as hydrogen peroxide, be added.

On Bach and Chodat's hypothesis, there are present in the potato oxygenase, peroxidase and peroxide; the peroxidase transfers oxygen from the peroxide to the guaiacum, and the oxygenase re-oxidizes the reduced peroxide. This may be termed the direct action.* On the other hand, in the cucumber juice, only peroxidase is present, so that in order to obtain the blue reaction with guaiacum, hydrogen peroxide, or other peroxide, must be added. This is the indirect action.

This idea has been accepted by Palladin,† who considers that normal respiration depends upon the presence of an oxidizable substance, oxygenase and peroxidase.

Peroxidases can practically always be found in living plant members, but the oxygenases are less stable and are quickly decomposed, giving origin to some of the respiratory carbon dioxide. The amount of these enzymes varies with the stage of development of the plant; thus in the embyro, oxygenase is at its minimum, but increases with the development of the plant and then diminishes as the growth of the organ ceases.

On the other hand, according to Porodko,‡ oxidases play scarcely any part in respiration.

The views of Bach and Chodat are not universally held; thus Moore and Whitley,§ as a result of a number of experiments, have arrived at the conclusions that the sole difference

* Wheldale: "Proc. Roy. Soc., Lond.," B., 1911, **84**, 121.
† Palladin: "Ber. deut. bot. Gesells.," 1906, **24**, 9.
‡ Porodko: "Beih. Bot. Centrbl,, 1904, **16**, 1.
§ Moore and Whitley: "Biochem. Journ.," 1909, **4**, 136.

between the various plant extracts, etc., which show an oxidizing action, consists in the presence of a small variable amount of peroxide which is chemically unstable. Juices possessed of such oxidizing properties have one type of ferment, a peroxidase, which acts only in the presence of peroxide, which, if not present in the natural extract, must be added. There is no proof of the existence of any other type of enzyme, such as oxygenase, engaged in oxidation processes. Thus the oxidases are brought into line with hydrolytic enzymes concerned in the phenomena of digestion, etc. :—

| | Hydrolysis. | Oxidation. |
|---|---|---|
| Substrate | Carbohydrates, fats, proteins | Oxidizable substances, e.g. phenols and chromogens |
| Combining body ("combinate") | Water (finally) | Oxygen which is yielded by hydrogen peroxide or organic peroxides |
| Catalyst | Diastase, zymase, etc. | Peroxidase, tyrosinase, etc. |

They further point out that any substance containing a peroxide linkage will activate a peroxidase just as any type of acid or alkali, which increases hydrogen or hydroxyl ion concentration, will activate a hydrolytic enzyme.

The reason why a plant extract containing oxidases will no longer give the guaiacum reaction when heated to 60° is that the peroxide, originally present in the juice, is destroyed, but not the peroxidase. So that although the heated juice is inactive, its oxidizing activity can be restored by the addition of a peroxide.

The work of Moore and Whitley is corroborated by Wheldale,* who finds that the power of the direct action, but not the indirect, is accompanied by the formation of a brownish pigment when the part is injured or subjected to the action of chloroform vapour. This action is common in the Compositæ, Umbelliferæ, Labiatæ, Boraginaceæ, and certain genera of the Scrophulariaceæ, Rosaceæ, Leguminosæ, and Ranunculaceæ. In the Cruciferæ, Caryophyllaceæ, Crassulaceæ and Ericaceæ

* Wheldale; "Proc. Roy. Soc., Lond.," B., 1911, 84, 121.

the action is either absent or very rare. In such cases she finds the direct action to be due to pyrocatechin which, on exposure to air, rapidly oxidizes and then acts as an organic peroxide, thus enabling the peroxidase, which is almost universally present, to transfer oxygen to the oxidizable substance.

From later investigations this same author * concludes that the direct oxidase reaction is due to the presence of a peroxidase (which will blue guaicum only in the presence of hydrogen peroxide) and an aromatic substance having a catechol grouping. On injuring the tissues, the peroxidase activates the oxidation of the aromatic compound with the formation of a peroxide. The system thus formed, peroxide—peroxidase, will then blue the guaicum. The presence of tannins may inhibit or mask the reaction.

Reed † finds that oxidases in general when purified to the extreme limit still give the catalase reaction, i.e. split hydrogen peroxide into water and oxygen, and that the peroxidase reaction is quite independent of the amount of hydrogen peroxide decomposed. He believes that the peroxidase combines with oxygen from hydrogen peroxide or from oxygenase to form an intermediate compound, which is a more potent oxidizing agent than the original source of oxygen. It is this intermediate compound which effects the final oxidation changes. Since in certain plants, e.g. the pine-apple, the peroxidase reaction is independent of any ability to decompose hydrogen peroxide, no catalase is present in certain stages of development of the fruit, he concludes that the substances which bring about the decomposition of hydrogen peroxide are not necessarily concerned with the enzymes which accelerate peroxide oxidations.

According to Ewart ‡ there is no justification for the distinction drawn between oxidase and peroxidase since their supposed fractional precipitation by means of alcohol is merely due to the attenuation of the solution. The organic oxidases are proteins which may or may not be combined with metals. They vary according to their strength; a strong solution will bring about direct oxidation from the oxygen held in solution, whilst the weak can only transfer oxygen from compounds

* Onslow: "Biochem. Journ.," 1919, **13**, 1.
† Reed: "Bot. Gaz.," 1916, **61**, 523; **62**, 53, 233, 303, 409.
‡ Ewart: "British Assoc. Rep.," 1915.

such as hydrogen peroxide, or dissolved oxygen in the presence of sensitizers such as sodium chloride.

In addition to oxidative processes carried out in the plant by these enzymes, they may play an important part in the preparation of raw food-material in the soil. Thus Schreiner and Reid † as the result of their experimental work have come to the conclusion that enzymes, principally peroxidases, are excreted by the roots, and so oxidize organic substances in the soil. Oxidation was found to be most potent in the root-hair regions and those places where the secondary roots are developing. The older parts of the root and also the root-cap do not produce these enzymes. The degree of the activity of the enzymes in the soil depends upon the character of the soil; thus the presence of sodium nitrate accelerates the oxidative processes, and, in brief, the most productive soils show the most vigorous oxidation. On the other hand, the reverse obtains in poor unproductive soils which contain substances which interfere with the process. These deleterious agents may be removed sometimes by suitable treatment, e.g. the use of absorbing agents such as lamp-black.

* Schreiner and Reid: "Bot. Gaz.," 1909, 47, 355. See also Schreiner and Sullivan: *id.*, 1911, 51, 156, 273.

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